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LAB IV: PAEDIATRIC NUTRITION, FOOD MICROBIOLOGY AND SANITATION & FOOD BIOTECHNOLOGY AND BIOSTATISTICS

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LAB IV: PAEDIATRIC NUTRITION, FOOD MICROBIOLOGY AND SANITATION & FOOD BIOTECHNOLOGY AND BIOSTATISTICS

SYLLABI

Block-I Paediatric Nutrition

1. Development of Low Cost Recipes
   a. Infants.
   b. Preschoolers.
   c. Elementary School Children.

Block-II Food Microbiology and Sanitation

1. Isolation of Microorganisms from Spoiled Food Sample.
2. Pure Culture and Preservation of Bacteria.
3. Gram Staining.
4. Motility Test.
5. Hydrolysis of Starch, Gelatin and Protein.
6. Identification of Prepared Slides
   a. Mould - Mucor, Rhizopus, Aspergillus, Penicillium, Yeast.
   b. Bacteria - Bacilli.

Block-III Food Biotechnology

1. Determination of pH of Different Foods using pH Meter.
2. Study Quality Characteristics of Foods Preserved by Drying/Dehydration/ Freezing.
3. To Perform Pasteurization of Fluids using Different Methods.
4. To Perform Blanching of Different Plant Foods.
5. Methods of Food Sampling and Concept of Shelf Life of Different Foods.

Block-IV Biostatistics

1. Tabulation and Graphical Representation of Data.
2. Calculation of Mean, Median and Mode.
3. Calculation of Standard Deviation, Standard Error and ANOVA (One Way and Two Way).
4. Chi-Square Test, t-Test, Regression and Correlation.

INTRODUCTION

NOTES

Paediatric nutrition is the maintenance of a proper well-balanced diet consisting of the essential nutrients and the adequate caloric intake necessary to promote growth and sustain the physiologic requirements at the various stages of a child development. Paediatric nutrition includes the nutritional needs of infants, children, and adolescents, i.e., nutrition for children from infancy up through 18 years of age. Due to lack of nutrition especially during critical periods of growth, results in improper development or illness, such as anaemia from deficiency of iron or scurvy from deficiency of vitamin C. Nutrition plays a major role in childhood development. Proper nutrition helps for proper growth and development of children. **Food microbiology** is the study of the microorganisms that inhibit, create, or contaminate food. This includes the study of microorganisms causing food spoilage; pathogens that may cause disease (especially if food is improperly cooked or stored); microbes used to produce fermented foods, such as cheese, yogurt, bread, beer, and wine; and microbes with other useful roles, such as producing probiotics. **Food safety** includes the processing and regulatory issues related to the role of microorganisms in food processing and preservation. The Hazard Analysis Critical Control Points (HACCP) is used to prevent contamination of food, equipment, and personnel. Food safety is a major focus of food microbiology. Numerous agents of disease and pathogens are readily transmitted via food which includes bacteria and viruses. Microbial toxins are also possible contaminants of food. However, microorganisms and their products can also be used to combat these pathogenic microbes. Probiotic bacteria, including those that produce bacteriocins can kill and inhibit pathogens. Alternatively, purified bacteriocins, such as nisin can be added directly to food products. Finally, bacteriophages, viruses that only infect bacteria can be used to kill bacterial pathogens. Thorough preparation of food, including proper cooking, eliminates most bacteria and viruses. However, toxins produced by contaminants may not be liable to change to non-toxic forms by heating or cooking the contaminated food. **Food biotechnology** is the use of technology for the determination of pH of different foods using pH meter, studying quality characteristics of foods preserved by drying/dehydration/ freezing, performing pasteurization of fluids using different methods, performing blanching of different plant foods, and using methods of food sampling and concept of shelf life of different foods. **Biostatistics**, also known as biometry, are the development and application of statistical methods to a wide range of topics in biology. It encompasses the design of biological experiments, the collection and analysis of data from those experiments and the interpretation of the results. Data collection methods must be considered in research planning, because it highly influences the sample size and experimental design. Data collection varies according to type of data. Data can be represented through tables or graphical representation, such as line charts, bar charts, histograms, scatter plot. Furthermore, measures of central tendency and variability can be very useful to describe an overview of the data. The correlations between two different kinds of data could be inferred by graphs, such as scatter plot.

This book, *Lab. IV: Paediatric Nutrition, Food Microbiology and Sanitation & Food Biotechnology and Biostatistics*, focuses on the various significant aspects of Paediatric Nutrition, Food Microbiology, Food Safety, Sanitation, Food Biotechnology and Biostatistics.

BLOCK I
PAEDIATRIC NUTRITION

NOTES

1. Development of Low Cost Recipe for Infants

Infancy is a period of the first year of life, in which focus point is growth and development. The skeletal growth, muscular development and fine motor skills are the salient features of this phase. In growth and development of the child nutrition plays a prominent role. The first 0-6 month nutritional requirement provided by breast milk. Breast milk is considered as a complete food since it provides both macro and micro nutrients. Exclusive breast feed should be emphasized at least for 6 month which helps in adhere the immunity of the child. During 6-12 months, supplementary feeding becomes essential, as energy requirement increases. After 6 months the complementary feeding should be introduced with breast milk to provide macro and micro nutrients.

Recommended Food Intake for Infants

Nutrient	**Age**	
	0-6 Month (5.4kg)*	**6-12 Month (8.4kg)***
Energy (kcal/kg/day)	92	80
Protein (g/kg/day)	1.6	1.69
Fat (g/day)	-	19
Calcium (mg/day)	500	500
Iron (mg/day)	46	5
Vitamin A (Retinol) (μg/day)	350	350
Beta-Carotene (μg/day)	-	2800

First Foods for Infants

From around six months, babies need solid food in addition to breast milk or formula, for adequate nutrients and energy. Introducing solid food at this age is also important to help infants develop skills required for eating, such as chewing.

Food given to infants in long day care should be nutritious and of appropriate textures as per their age and developmental stages. The daily menu should contain a variety of age-appropriate nutritious foods each day to provide energy and nutrients.

Iron

Nutritious foods that contain iron should be among the first foods introduced to an infant. Iron-rich foods include iron-fortified infant cereals, pureed meat, poultry and fish, egg, cooked plain tofu and legumes, for example chickpeas, red, green

or brown lentils, split peas and beans, for example kidney beans, navy beans, broad beans, moong beans.

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Other nutritious foods can be introduced in any order and at any rate that suits the infant. Although cow's milk products (including full-fat yoghurt, cheese and custard) may be given in the food and not as a main drink before the age of 12 months. By 12 months of age infants needs a variety of nutritious foods. Breast milk or infant formula should be continued while introducing solids.

Food Textures and Consistencies for Age and Developmental Stage

Increasing and varying food texture for infants is essential for their oral motor development, for example learning skills required for eating, such as chewing and to help them accept different food textures. It is important to always offer foods have appropriate texture and consistency for infant's developmental stages. This means progressing quickly through the puree/mashed phase and offering foods with varied textures. If foods are pureed or mashed, for example soft vegetables, they should not be mashed together but should be presented individually either in pieces or on a spoon to encourage infants to taste and accept individual flavours.

The following can be used as a guide for preparing foods of appropriate texture for infants:

- From around 6 months – offer coarsely pureed/mashed foods, progressing to lumpy and finely chopped options.
- By 8 months – offer chopped and finger foods to encourage children to start feeding themselves.
- By 12 months – offer foods from the regular menu with a variety of tastes and textures in children's size portions.

Drinks for Infants from Birth to One Year and Beyond

Breast milk or infant formula should be the main drink in the first 6 months of life. From around 6 months, small amounts of cooled, boiled tap water can supplement breast milk or infant formula. After 12 months, water and full cream cow's milk should be the main drinks offered. Tap water is an important source of fluoride for young children. Clean and safe tap water should be given.

Low fat and reduced fat milks are not recommended in the first 2 years of life, but are suitable for children over the age of 2 years.



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What Not to Include on the Menu for Infants

When providing food and drinks for infants it is important to keep the following in mind:

- Avoid giving foods with a high risk of choking. Always supervise children during meal and snack times.
- Cow's milk should not be given as the main drink before 12 months of age.
- Salt and sugar should not be added to infant's food. Offering sweet and salty foods in early childhood can result in children developing a preference for these foods which may be carried into later life.
- Infants should not be given foods with high levels of saturated fat, added sugar and/or added salt, for example cakes, biscuits and chips.
- Honey should not be given to babies, as it may contain bacterial spores that can cause infant botulism if given to babies under 12 months of age.
- Sweet drinks like fruit juice and fruit drinks, flavoured milk, soft drinks and cordials should not be given to infants and children. These drinks add sugar to the diet and can increase the risk of children becoming overweight and developing oral health problems.
- Tea (including herbal tea) and coffee are not appropriate drinks for infants and children.

FOODS, DRINKS AND TEXTURES FROM BIRTH TO 1 YEAR

From Birth to Around 6 Months

Liquids only that includes breast milk and infant formula.

Nutrient Requirements of 6 Months to One Year Old Infant

Following are the nutritional components recommended in baby food for 6 months old:

Calcium: Calcium in food is essential for their bone and teeth development.

Iron: Iron helps carry oxygenated blood to all the developing parts of the body.

Zinc: Zinc helps cell repair and growth.

Fat: Fat insulates the baby and helps in brain development.

Carbohydrates: Carbohydrates are the primary source of energy and fuels their day-to-day activities.

Protein: These are a must for growth as they act as building blocks for cells.

Vitamins: Different vitamins contribute to the growth of the baby differently, and almost all the vitamins including Vitamin A, B1, B2, B3, B6, B12, C, D, E and K are essential to the baby.

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Minerals: Minerals like sodium and potassium directly influence the growth of the baby.

Around 6 Months to 8 Months

Mashed, progressing to minced and lumpy food as following:

- Breast milk, infant formula cooled boiled tap water (as required).
- Iron-fortified infant cereals.
- Mashed tofu/beans/chickpeas.
- Mashed hard boiled or scrambled egg.
- Mashed cooked vegetables, for example pumpkin, potato, sweet potato, carrot, broccoli, cauliflower, peas and spinach. Ensure these are not mashed together.
- Rice and semolina.
- Yoghurt, custard, cow's milk in food (not as a drink).

8 Months and Beyond

Grated, diced, chopped, finger foods as following:

- Breast milk, infant formula and cooled boiled tap water.
- Diced tofu and cooked lentils.
- Sliced hard boiled eggs or scrambled eggs.
- Pieces of soft cooked vegetables, for example potato, pumpkin, sweet potato, carrot, green beans and broccoli.
- Diced soft fruit, for example peach, mango, pear, avocado and melon.
- Grated apple, ripe banana pieces and sliced watermelon.
- Yoghurt (full fat), custard, cottage/ricotta/cream cheese and grated cheese.
- Grain (cereal) foods, such as bread, toast, oats, pasta, noodles and rice.
- Cow's milk in food.



12 Months and Beyond

Varieties of textures can be given as following:

- Breast milk, cow's milk as a drink, water.
- Eggs – boiled, poached or scrambled.
- Continue above vegetables (mentioned above in 8 months and beyond age group) and start adding celery, cucumber, tomato, capsicum, mushrooms, cooked cabbage and cooked sprouts.
- Continue above fruits (mentioned above in 8 months and beyond age group) and start adding seedless grapes (cut in quarters with skins removed), orange (membrane and pips removed), kiwi fruit and pineapple.
- Cheese sticks or slices and cream cheese.
- Soft cracker biscuits, pikelets, pasta (differing shapes, noodles and spaghetti cut up).
- Bite-sized sandwiches with moist fillings.

LOW COST RECIPE FOR INFANTS

Following are some low cost recipes for infants:

A. Banana, Apple and Rice Kheer (Per 100 g)

Ingredients

Rice 15 g

Banana 80 g

Apple 150 g

Milk 100 ml

Jaggery 15 g





Method of Preparation

1. In a saucepan, add rice and water.
2. Keep gas (stove) heat on high and wait for boil to come.
3. Reduce heat to medium low and cook for 15 minutes.

NOTES

4. After 15 minutes, cut the apple in uniform pieces, add and cook for another 10 minutes.
5. Turn off the heat and allow to cool.
6. Put the prepared mixture on blender.
7. Add banana with the mixture and blend until desired consistency.
8. It is ready to be served.

B. Wheat Gram Porridge

Ingredients

Roasted Wheat Flour 25 g

Roasted and Powdered Bengal Gram 15 g

Roasted and Powdered Groundnut 10 g

Sugar or Jaggery 30 g

Spinach (or Any Green Leafy Vegetable) 30 g



Method of Preparation

1. Roast groundnut, wheat and Bengal gram and powder them separately.
2. Mix wheat, Bengal gram and groundnut powders and prepare a batter by adding jaggery.
3. Dissolve the prepared batter in a suitable amount of water and make a thin syrup.
4. Boil spinach in water until soft.
5. Mash the spinach and strain through a clean cloth.
6. Add the vegetable juice to the batter and cook for a few minutes with continuous stirring till semi-solid.
7. It is ready to be served.

NOTES

C. Suji Porridge

Ingredients

Wheat Rava (Suji) 40 g

Green Gram Dal (Washed) 10 g

Jaggery 20 g

Fat Oil 10 g

Cardamom 1 pc.

Water 200 ml



Method of Preparation

1. Clean wheat rava and green gram dal.
2. Roast separately and mix.
3. Boil water and add the above mixture in it.
4. Cook till it becomes soft.
5. Add powdered jaggery and continue cooking, till jaggery dissolves.
6. Add oil and cardamom powder mix well and serve hot.

Nutritive Values Per 100 gms

Calories 340

Protein 7.2 g

Iron 1.66 mg

Carotene 4.9 μg

D. Dalia Khichri

Ingredients

Dalia 100 g

Masur Dal 30 g

Moong Dal 20 g

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Potato 50 g

Pumpkin 50 g

Amaranth (Tender) 50 g

Onion 10 g

Oil 10 ml

Salt and Haldi as per Taste

Water Sufficient to Cook



Method of Preparation

1. Wash and peel the vegetables.
2. Cut the vegetables into pieces (medium size).
3. Wash green leafy vegetables thoroughly and chop.
4. Wash dalia and dal.
5. Heat oil in a pan and add chopped onion and vegetables.
6. Fry for some time.
7. Add dalia and dal, mix thoroughly.
8. Add salt, haldi powder and warm water
9. Cook till dalia and vegetables become soft.

Nutritive Values Per 100 gms

Calories 220

Protein 8 g

Iron 3.19 mg

Carotene 884 µg

E. Rice Food Mix

Ingredients

Rice 75 g

Moong Dal 25 g

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Method of Preparation

1. Roast rice and moong dal separately.
2. Grind rice and moong dal to a fine powder.

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3. Mix the dry powder.
4. In a saucepan add water and keep gas (stove) heat on high and wait for water to boil.
5. Add the mixed powder and reduce heat to medium low and cook for 15 minutes or until a thick paste is made.
6. This mixed powder can also be stored for later instant use by filling it in an air-tight bottle/container.

Nutritive Value Per 100 gms

Calories 346

Protein 11.2 g

Iron 1.5 mg

Carotene 12 µg

F. Maize Mix

Ingredients

Roasted Maize Powder 50 g

Roasted Moong Dal Powder 20 g

Roasted Til Powder 10 g

Sugar (Powdered) 20 g







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Method of Preparation

1. Roast maize, moong dal, til separately.
2. Grind maize, moong dal, til and sugar separately to a fine powder.
3. Mix the dry powder.
4. In a saucepan add water and keep gas (stove) heat on high and wait for water to boil.
5. Add the mixed powder and reduce heat to medium low and cook for 15 minutes or until a thick paste is made.
6. This mixed powder can also be stored for later instant use by filling it in an air-tight bottle/container.

Nutritive Value Per 100 gms

Calories 377

Protein 12 .28 g

Iron 2 .87 mg

Carotene 61 µg

2. Development of Low Cost Recipes for Preschoolers

Generally, by the time the child is I to 1.5 year's old, breast milk is not available. Therefore the child have to depend on other foods. By this age the child have cut teeth and know to eat by itself and has access to the adult type of foods. But as adult foods are mostly based on rice, wheat or other cereals. They are relatively low in proteins. As the child requires more nutritious foods that can provide more protein and calories. Therefore, in the post-weaning period, proper nutritional care of the child is essential to ensure normal growth not only then but even in later life.



NOTES

Recommended Dietary Allowances for Childhood

Nutrient	
Energy (kcal)	1350
Protein (g)	20.1
Visible Fat (g)	25
Calcium (g)	600

Principles Governing the Formulation of the Recipes

A pre-school child requires daily about 20 g. of protein and about 800-1500 calories. The additional protein needed can be given by protein-rich foods like milk, meat, fish and egg or pulses. Nuts and oilseeds are also good sources of proteins and calories. The cereals and millets commonly used in various regions of our country are rice, wheat, jowar, bajra, ragi and maize etc. Cereals in general provide about 350 calories per 100 g. They are however, relatively poor sources of protein, the content varying from about 77 in rice to about 12% in wheat. Pulses are about the richest natural sources of proteins. They contain about 22 to 25% protein and like cereals. A growing child requires apart from protein and calories, other nutrients such as calcium and vitamin A. Appropriate use of foods like green leafy vegetables and milk takes care of these requirements and so these foods have been included in some of the formulations.

Helpful Feeding Information for Your Preschooler

Preschool-age children (ages 3 to 5) are still developing their eating habits and need encouragement to eat healthy meals and snacks. These children are eager to learn. They will often imitate eating behaviours of adults. They need supervision at mealtime as they are still working on chewing and swallowing skills.

These are some helpful mealtime hints for preschool-age children:

- Make meals, give regularly scheduled snacks, and limit unplanned eating.
- Discourage poor behaviour at mealtime. Focus on eating, not playing with food, or playing at the dinner table.
- Running or playing while eating can cause a child to choke. Have your child sit when eating.
- Keep offering a variety of foods. Have the attitude that, sooner or later, your child will learn to eat almost all foods.
- Provide examples of healthy eating habits. Preschoolers copy what they see their parents doing. If you have unhealthy eating habits, your child will not learn to eat healthy.

Healthy Food Choices

Following are the required food that can provide a good nutrition to a preschooler:

- **Grains:** Foods that are made from wheat, rice, oats, cornmeal, barley, or another cereal grain. Examples include whole-wheat, brown rice, and oatmeal.

NOTES

- **Vegetables:** Vary your vegetables, choose a variety of colourful vegetables which includes dark green, red, and orange vegetables, legumes (peas and beans), and starchy vegetables.
- **Fruits:** Any fruit or 100% fruit juice. Fruits should be fresh, dried or pureed.
- **Dairy:** Milk and milk products that are fat-free or low-fat products, as well as those that are high in calcium.
- **Protein:** Low-fat meats and poultry. Vary your protein routine, for example choose more fish, nuts, seeds, peas, and beans.



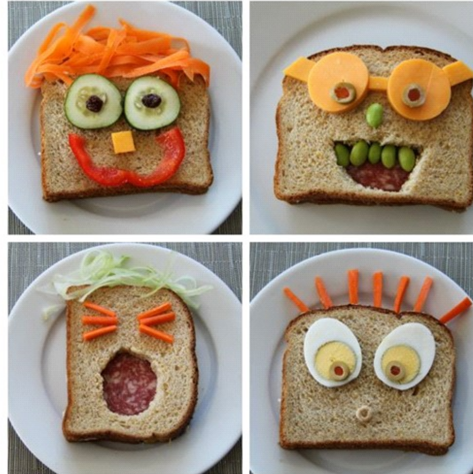
Some nutritious and delicious snack can also be given to preschooler as follows:

- Sandwich
- Well-cooked vegetables and low-fat dip
- Whole grain crackers and cheese
- Yogurt
- Fruit smoothies or fruits
- Milk
- Egg - hard-boiled or scrambled egg
- Dry cereal or with milk
- Low-fat microwave popcorn (starting at age 4).





NOTES





A. Jowar Upma

Ingredients

Broken Jowar 45 g

Roasted Groundnuts 15 g

Oil 7 g

Cumin Seeds ½ tbsp.





Method of Preparation

- Put oil in a pan, add cumin seeds when oil is heated.
- When cumin seeds start crackling add broken jowar and roast until they are slight brown.

- Add water in the pan and cook till it becomes soft.
- Add roasted groundnuts and cook for few more minutes.

B. Bajra Khichdi

Ingredients

Bajra 45 g

Green Gram Dal 25 g

Carrot 20 g

Oil 8 g

Cumin Seeds ½ tbsp

Sweet Potato can also be used in place of Carrot





Method of Preparation

- Grate carrot and keep aside.
- Take a saucepan and boil dal until half done.
- Add bajra and grated carrot in the boiled dal.
- Now cook till the grains become soft.
- Remove from the gas (stove) and season with cumin seeds and oil.

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C. Dal Vegetable Soup

Ingredients

Green Moong Dal 1tbsp

Chopped Tomatoes ¼ cup

Chopped Cabbage ½ cup

Chopped Spinach ½ cup

Salt (as per Taste)



Method of Preparation

1. To make dal vegetable soup combine all ingredients along with 1 cup of water in a pressure cooker and pressure cooker for 2-3 whistles.
2. Allow the steam to escape before opening the lid. Keep aside until cool.
3. Once cooled blend it and make a smooth puree.
4. Transfer the puree to a saucepan and add salt as per taste. Mix well and bring to a boil.
5. Ready to serve.

D. Sprouts Khichdi

Ingredients

Mixed Sprouts (Moong, Channa, etc.) 2 tbsp

Rice Soaked (for 15 Minutes and Drained) 2 tbsp

Ghee ½ tbsp

Cumin Seeds ¼ tbsp

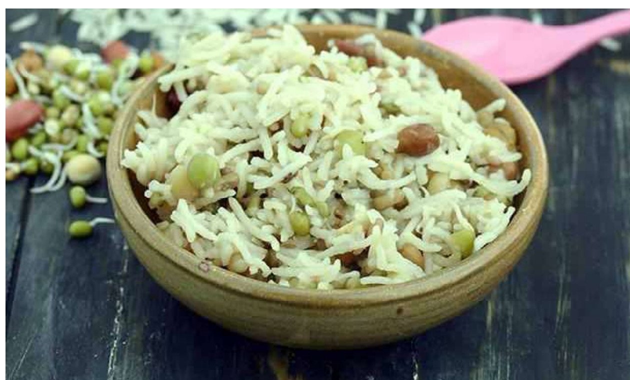
A Pinch of Asafoetida (Hing)

Garlic Paste ½ tbsp

Onion Finely Chopped 1 tbsp

Salt as per Taste

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Method for Preparation

1. To make sprouts khichdi, heat ghee in a pressure cooker and add cumin seeds.
2. When cumin seeds crackle, add the asafoetida and garlic paste and saute on a medium flame for a few seconds.
3. Add onions and saute on a medium flame for 30 seconds.
4. Add rice and sprouts and saute on medium flame for 30 seconds.
5. Add ¾ cup of water and salt, mix well and pressure cook for 3 whistles.
6. Allow the steam to escape before opening the lid.
7. Mash the khichdi evenly.
8. Serve the sprouts khichdi with curd for better taste.

3. Development of Low Cost Recipes for Elementary School Children

Proper nutrition for a healthy child in this age group should provide adequate essential nutrients, fiber and energy; sufficient enough to maintain proper growth, maximize cognitive development and promote health. It should introduce balance among the foods consumed, such that foods rich in some nutrients do not displace foods that are rich in other nutrients. Balance within the diet helps to ensure adequate nutrient intake within the diet. A child's diet should provide sufficient energy intake for proper growth and development while preventing excess weight gain. To do so, foods selected should be high in nutrient density, meaning the food should have a high nutrient to calorie ratio. The diet should be moderate enough so not to deliver too much of a dietary constituent. It should also ensure variety using different foods on different occasions to ensure adequacy, balance and moderation among all nutrients needed to support the child's growth and developmental needs.

Nutrition Guidelines for School-Age Kids

As children develop, they require the same healthy foods adults eat, along with more vitamins and minerals to support growing bodies. This means whole grains (whole wheat, oats, barley, rice, millet, quinoa); a wide variety of fresh fruits and vegetables; calcium for growing bones (milk, yogurt, or substitutes if lactose intolerant); and healthy proteins (fish, eggs, poultry, lean meat, nuts, and seeds).

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Vegetables: 3-5 servings per day. A serving might be one cup of raw leafy vegetables, 3/4 cup of vegetable juice, or 1/2 cup of other vegetables, raw or cooked.

Fruits: 2-4 servings per day. A serving may consist of 1/2 cup of sliced fruit, 3/4 cup of fruit juice, or a medium-size whole fruit, such as an apple, banana or pear.

Whole Grains: 6-11 servings per day. Each serving should equal one slice of whole grain bread (beware of breads with only "Wheat" versus "Whole Wheat/ Whole Grain", 1/2 cup of rice or 1 ounce of whole grain cereal (Do not use the high sugar cereals).

Protein: 2-3 servings of 2-3 ounces of cooked lean meat, poultry, or fish per day. A serving in this group may also consist of 1/2 cup of cooked dry beans, one egg, or 2 tablespoons of peanut butter for each ounce of lean meat.

Dairy Products: 2-3 servings (cups) per day of low-fat milk or yogurt, or natural cheese (1.5 Ounces = One Serving).

Zinc: Studies indicate that zinc may improve memory and school performance, especially in boys. Good sources of zinc are oysters, beef, pork, liver, dried beans and peas, whole grains, fortified cereals, nuts, milk, cocoa, and poultry.

Water: 6-8 glasses each day.

Healthy Fat: Healthy fats are also important to a child's development. These include monounsaturated fats from plant oils like canola oil, peanut oil, and olive oil, as well as avocados, nuts (like almonds, hazelnuts, and pecans), and seeds (such as, pumpkin, sesame; and polyunsaturated fats, including Omega-3 and Omega-6 fatty acids, found in fatty fish, such as salmon, herring, mackerel, anchovies, and sardines, or in unheated sunflower, corn, soybean, and flaxseed oils, and walnuts.

A. UPMA – Sooji and Vegetables

Ingredients

Sooji 40 g

Oil 15 ml

Tomatoes 20 g

Potato 10 g

Green Peas 10 g

Onion 20 g

Coriander Leaves 5-6

Curry Leaves 5-6

Carrot 10 g

Black Gram Dal (Washed) 10 g

Coriander Powder ¼ tbsp

Mustard Seeds ½ tbsp

Water 80 ml

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Method of Preparation

1. Soak black gram dal in water for 10 minutes.
2. Wash and chop all the vegetables into small equal size pieces.
3. Take a pan and heat oil.
4. When the oil is heated add mustard seeds, black gram dal and curry leaves till they splutter.
5. Now add chopped onions and cook until golden brown.
6. Add vegetables and cook they become soft.
7. Now add salt (as per taste) and coriander powder.
8. After that add sooji and water and cook till all the water is absorbed.
9. Garnish with coriander leaves and serve hot.

Nutritional Value Per 100 gms

Energy 164 kcal

Protein 3.4 g

Fat 8.3 g

Iron 0.5 mg

Calcium 27 mg

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B. Dal Palak

Ingredients

Arhar Dal (Washed) 50 g

Spinach 50 g

Oil 15 ml

Salt (as per Taste)

Turmeric Powder ¼ tbsp

Coriander Powder ¼ tbsp

Cumin Seeds ¼ tbsp.

Water (as per Requirement)



Method of Preparation

1. Clean and wash dal.
2. Soak the dal for 1 hour in clean water.
3. Wash spinach thoroughly and chop it finely.
4. Now take a pan add water, dal, spinach and spices. Boil them until dal is cooked.
5. Now take a pan and heat oil and add cumin seeds.
6. When cumin seeds starts to crackle add the cooked dal in it.

Nutritional Value Per 100 gms

Energy 158 kcal

Protein 6 g

Fat 8 g

Iron 0.9 mg

Calcium 36 mg

C. Rawa Porridge

Ingredients

Rava (Suji) 50 g

Green Gram Dal (Moong dal) 15 g

Jaggery (Powdered) 20-25 g

Ghee 10 g

Water 250 ml



Method of Preparation

1. Clean wheat rava and green gram dal.
2. Roast them separately and mix.
3. Boil water in a pan.
4. Add rava and green gram dal and cook until they becomes soft.
5. After it is cooked add jaggery and cook till it dissolves.
6. Add ghee and flavourings of choice like green cardamom powder (as per choice).
7. Mix well and serve hot.

Nutritive Values Per 100 gms

Energy 349 kcal

Protein 7.29 g

Iron 1.66 mg

Carotene 4.9 µg

D. Nutritious Khichdi

Ingredients

Rice 100 g

Dal 50 g

Potatoes 20 g

Beans/Dal/Chana/Peas 20 g

Spinach/Green Leafy Vegetable Locally Vegetable 50 g

Tomatoes 20 g

Onion 10 g

Coriander Leaves 10 g

Cooking Oil 15 g

Salt, Chilies, Turmeric Powder (as per Taste)

Jeera/Ajwain ½ tbsp.



Method of Preparation

1. Wash all the vegetables and dry in a basket.
2. Chop all vegetables.
3. Wash and soak rice and dal separately for 30 minutes.
4. Heat oil in a pan. When the oil is heated add jeera/ajwain. Add onions when jeera starts crackling and fry the onions till they become golden brown.
5. Add all the vegetables and spices and cook for 1-2 minutes.
6. Now add soaked rice and dal in it.
7. Add sufficient water and cook until soft.
8. Prepare the consistency as per the taste.
9. Garnish with coriander leaves.

Nutritive Value Per 100 gms

Calories 216.2

Protein 6.13 g

Iron 4.68 mg

Carotene 699 µg

E. Vegetable Pancake

Ingredients

Wheat Flour 100 g

Besan 50 g

Tomatoes 100 g

Spinach / Green Leafy Vegetable of Choice 100 g

Coriander Leaves 50 g

Onion 100 g

Ghee 50 g

Salt, Red Chillies Powder, Haldi (Turmeric Powder) (as per Taste)



Method of Preparation

1. Wash all vegetables thoroughly and chop them into small pieces.
2. Mix pancake base (wheat flour and besan) in a pan and prepare a batter.
3. Add all chopped vegetables and spices in the batter.
4. Make sure that the batter is of pouring consistency.
5. Heat griddle and grease it with a little ghee.
6. Place the batter on the griddle and spread evenly in circular motion.
7. Apply ghee from the sides and cook on both sides until golden brown.
8. Serve hot.

Nutritive Values Per 100 gms

Energy 186 kcal

Protein 4.41 g

Iron 5.32 mg

Carotene 1896 μg

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Practical Assignments

1. Given below is the list of recipes; develop your own method of cooking and standardize it. Record the methodology and nutritive value in the given format:
   a. Halwa
   b. Laddoo
   c. Pulao
   d. Nutritious Roti
   e. Firni

Name of the Recipe	
Ingredients	**Amount**
Methodology (Mention Special Cooking Instructions, if Needed)	
Nutritive Values Per 100 gms	

2. Prepare instant food mixes (any one from the given list and record information in the format given below)
   a. Puffed Rice Mix
   b. Millet Mix

Name of the Mix	
Ingredients	**Amount**
Methodology (Mention Special Cooking / Storage Instructions, if Needed)	
Nutritive Values Per 100 gms	

BLOCK II
FOOD MICROBIOLOGY AND SANITATION

NOTES

Microorganisms found in food can have either a negative or positive effect; some cause spoilage of foods, others can cause foodborne illness, and some can transform food in a beneficial way (food fermentation). Microorganisms have specific growing requirements that include nutrients, environmental temperature, presence/absence of oxygen, pH, moisture, etc. Therefore, changes in these factors will determine which microorganisms will survive and colonize a food product.

Escherichia coli (E coli)

Escherichia coli bacteria are environmental microorganisms that have been isolated from human and animal intestines, and are considered an important part of a healthy human intestinal tract. These bacteria were discovered in the colon of humans in 1885 by Dr. Theodor Escherich, who named it *Bacterium coli commune*. It was not until 1954 when *Escherichia coli* name was recognized. *Escherichia coli* is a large group of bacteria, of which most are harmless and have been broadly used in microbiology laboratories in research and food safety purposes. Pathogenic *Escherichia coli* can cause a broad range of human diseases that span from gastrointestinal tract to urinary tract, bloodstream, and central nervous system. Certain serotypes of *Escherichia coli* produce a cytotoxin known as Shiga toxin (Stx). Organisms from this group are named Shiga Toxin producing *Escherichia coli* (STEC). Some strains of STEC are associated with Hemorrhagic Colitis (HC) and Hemolytic Uremic Syndrome (HUS) in humans; this last group is called EnteroHemorrhagic *Escherichia coli* (EHEC). *Escherichia coli* is the main EHEC serotype associated with foodborne outbreaks.

Microorganisms Associated with Food Fermentation

Lactic Acid Bacteria (LAB)

Lactic Acid Bacteria (LAB) play an important role in the food industry, that is isolated from fermented vegetable foods, beverages, dairy products, and meats; however, they can also be responsible for spoilage of food. LAB have been used as starter culture in food fermentation, contributing to food preservation, taste and texture. *Streptococcus*, *Enterococcus*, *Lactococcus*, *Lactobacillus*, and *Leuconostoc* are all LAB. They are fastidious organisms that need many vitamins, amino acids, purines, and pyrimidenes for their growth; they normally depend on sugar fermentation for energy. LAB are generally recognized as facultative anaerobes. They grow under slightly acidic conditions, when pH is between 4.5 and 6.4.

Yeast

Yeasts are associated with fermented foods, such as bread, alcoholic beverages, rice, soy sauce, olives, red hot chili pepper, and cheese. Yeast alone, or in

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combination with LAB and/or molds have a significant effect on food quality parameters, such as taste, texture, and aroma. For isolation and cultivation of yeast, Potato Dextrose Agar/Broth, Brain Heart Infusion, Dextrose Agar, YM media, etc. are recommended.

Candida has been identified as the primary yeast involved in the natural fermentation of some food materials. Within this genus, *Candida glabrata* was isolated from fermented cereal based beverages and food products. *Candida krusei* was previously isolated from cucumbers and is an important starter culture in sourdough. *Candida norvegensis* has not been isolated from vegetable products but is important as a starter culture in sourdough bread. Additionally, *Rhodotorula spp.* has been isolated from red chili pepper mash, bread products, alcoholic beverages, and cucumbers. *Saccharomyces cerevisiae* has been isolated from several vegetable products, such as pickles, olives, cereal based products, alcoholic beverages, and is also an important producer of esters.

Detection of Microorganisms in Foods: Methods and Technique

Various techniques can be used for determination of microorganisms in food. Some of them give total count (viable and non-viable) while others give only viable count. Following are the methods for detection of microorganisms in foods:

1. Standard Plate Count (SPC)
    - Pour Plate Technique
    - Spread Plate Technique
    - Streak Plate Technique
2. Membrane Filter Technique
3. Most Probable Number (MPB) Method
4. Direct Microscopic Count (DMC)
5. Electronic Counter
6. Dye Reduction Test

Standard Plate Count (SPC)

Standard Plate Count gives viable count of organism present in food. The number of organisms in original food is determined by counting the colony on agar plate. Two major assumptions of SPC are:

- Microorganisms in suspension are separated as single cell so that each colony is developed from single cell.
- All viable cells placed on medium will multiply and produce a colony.

Incubation time and temperature for different microorganisms:

- *Psychrophile* 7°C, 7 days
- *Mesophile* 35°C, 24 to 48hrs
- *Thermophile* 55°C, 48hrs

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Advantages of SPC

Following are the advantages of SPC:

- It gives viable count.
- It is extremely sensitive, i.e., extremely low and high microbial population can be counted.

Disadvantages of SPC

Following are the disadvantages of SPC:

- If the suspension is not homogenous and contain aggregate of cells, the colony count will be lower than the actual number of microorganisms.
- If the suspension contains different types of microorganisms, all of them cannot grow in the same medium and under the same condition.

Types of Standard Plate Count (SPC) Method

There are two types of SPC methods as follows:

- Pour Plate Technique
- Spread Plate Technique
- Streak Plate Technique

Pour Plate Technique: In this method, food is firstly serially diluted in appropriate diluent. Then, measured volume of sample from diluted tube is placed in petri dish. Melted agar at 44-45^{o}C is mixed with it. After homogenous mixing of sample with melted agar, it is kept for solidification. Then the petri dish are incubated at appropriate time and temperature. Plate containing colonies between 30-300 is selected and number of colonies are counted. Now, number of organisms in original food sample is calculated by the following formula:

Colony Forming Units (CFU/ml) = (Number of Colonies/ Volume of Sample) x Dilution Factor

Since, Psychrophiles cannot survive temperature of melted agar, this technique is not suitable for them. In this method, both surface and subsurface colonies are developed. Subsurface colonies are difficult to be isolated.

Spread Plate Technique: In this method, appropriately diluted sample is placed on the surface of solidified agar. Then the drop of sample is spread over agar surface using bent glass rod. Plate is incubated for sufficient time and temperature, then number of colonies are counted. Calculation of number or organisms is done similarly as in pour plate technique. This method is suitable for Psychrophile also and only surface colonies are developed.

Streak Plate Technique: In this technique, a transfer loop is used to spread the specific volume of specimen over a surface of solidified agar. The transfer is done by calibrated loop of specific volume. Sometimes, selective and differential media can be used to select growth of specific organism.

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Membrane Filter Technique

Membrane filter technique is particularly important to analyse microorganisms in liquid food in which microbial content is too low. In this methods, major measured volume of liquid filtered through membrane filter of specific porosity. Then filter pad is removed and placed on the surface of agar plate and then incubated. Microorganisms grow on surface of membrane filter to form colony. Then total number of organisms in original sample is calculated. Nutrient or selective agar media can be used for microbial growth.

Most Probable Number (MPB) Method

Most Probable Number (MPB) Method is statistical technique to determine number of organisms in sample. It gives most probable number but not the actual number. Turbidity, gas production and acid production are observed to determine microorganisms. This method is based on 3 steps:

- Presumptive Test
- Confirmed or Confirmatory Test
- Completed Test

Direct Microscopic Count (DMC) Method

In Direct Microscopic Count (DMC) method, there is no difference between dead and viable cells. Total cells are counted. In this method, the result is obtained faster than most other methods because incubation period is not required. In case of liquid food, direct smear is made. For solid food, it must be first divided up to 10-1. Fatty foods must be defatted in xylene or acetone for preparation of smear. The xylene/acetone is then removed by dipping it in ethanol. In this method, number of microorganisms in microscopic field are counted directly. This technique is widely used to assess the quality of raw milk and other dairy product.

Example of Direct Microscopic Count (DMC) Method

Breed Count Method: It is an example of Direct Microscopic Count. This method was initially developed by R.S Breed. In this method, 0.01ml sample is spread over 1cm square area on slide. If sample is fatty, it should be defatted with xylene or acetone. Excess xylene or acetone is then removed by dipping it into ethanol. Then slide is dried, fixed and stained with appropriate dye and observed under microscope. Average number of microorganisms per field is counted. Then area of microscopic field is determined from which number of microorganisms in original sample is calculated. It is not practical to count entire field.

Advantages of DMC Method

Following are the advantages of DMC method:

- It is simple and rapid technique.
- Morphology as well as Gram reaction of microorganism spore production can be observed in microscope.

- Very small amount of sample is needed.
- The prepared slides can be stored and maintained as permanent record.

Disadvantages of DMC Method

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Following are the disadvantages of DMC method:

- DMC cannot distinguish viable and non-viable cells.
- Food particles are not always distinguishable from microorganism's cell.
- Some microorganisms do not take stain and may not be counted.
- It is very difficult to count microorganisms when the initial load is very high.

Electronic Counter Method

In electronic counter method, standard volume of suitable dilution of suspension is placed in electronic counter. The machine has small aperture through which microorganisms can pass. The passage of microorganisms through aperture causes alteration in electric resistance across it which is recorded as on impulses. These impulses are counted by suitable circuit in the counter. Number of impulses from fixed volume of sample is used to calculate number of organisms in original food sample.

Dye Reduction Test

Two dyes are commonly employed in dye reduction test to estimate viable number of organisms:

- Methylene Blue Reduction Test
- Resazurin Reduction Test (Rapid Test)

Methylene Blue Reduction Test

Methylene blue reduction test is commonly used to determine number of viable organisms in raw milk. In this method, methylene blue is mixed with raw milk or incubated. Microorganisms present in milk reduce methylene blue to form leucomethylene blue so that milk becomes blue to colourless. The time of decolourization of milk is indicative of number of viable organisms. If number of organisms are higher it is decolorized in shorter time and vice-versa. In this method microbial quality of milk assessed by reduction time.

Resazurin Reduction Test (Rapid Test)

It is an example of rapid dye reduction test use to determine number of viable organism in food, such as raw milk. In this test, Resazurin dye is mixed with raw milk. Microorganism present in milk reduce Resazurin such that its colour changed from stale blue to pink or colourless. If the number of microorganism is higher, dye is reduced in shorter time and vice-versa. Therefore, microbial load of milk can be predicted by reduction time of Resazurin. In this method, result is obtained within 10 minutes.

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Advantages of Dye Reduction Test

Following are the advantages of Dye Reduction Test:

- It is simple, easier, and inexpensive test.
- Only viable cells actively reduce the dye. So, that number of viable organism can be predicted.

Disadvantages of Dye Reduction Test

Following are the disadvantages of Dye Reduction Test:

- Not all microorganisms reduce the dye equally.
- They are not applicable for food that contain reducing substances, such as reducing enzymes unless special steps are employed.

EXPERIMENT 1: ISOLATION OF MICROORGANISMS FROM SPOILED FOOD SAMPLE

Aim and Objective

To isolate and identify the micro-organisms involved in the contamination of food.

Materials Required

Nutrient Agar Plates

Mixed Cultures of Bacteria

Inoculating Wire Loop

Needle and Spirit Lamp

Isolation of Bacteria

Isolation of bacteria forms a very significant step that involves various steps as follows:

- Specimen collection
- Preservation and transportation of specimen
- Microscopic examination of sample
- Various methods used for isolation of bacteria

Cleaning of Glass Wares and Sterilization

All glass wares utilized for this work were thoroughly washed with detergent rinsed with distilled water, it was dried and sterilized in the hot air oven at a temperature of 160°C for 1 hour.

Sterilization of Culture Media

The method of sterilization used in the work is the method of autoclaving. The majority of culture media are sterilized by being autoclaving. It is important to sterilize a medium at the cored temperature and for the correct length of time as instructed in the method of preparations under autoclaving can result man unsterile

medium which will need to be discarded over autoclaving and it can cause precipitation alternation of pH and the destruction of some essential components in a medium.

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Streak Plate Method of Separating Bacteria from a Mixture of Microorganisms

1. Label all the plates on the bottom.
2. Sterilize a wire loop on spirit lamp flame and allow it to cool.
3. Hold the wire loop in your right hand, pick bacterial growth from a well-isolated colony (for culture on solid agar) or dip the loop into a culture broth (for broth cultures) and withdraw a loopful of the culture.
4. Lift the Petri dish cover with the left hand and open by holding at an angle of 60 degree.
5. Place the loop containing the inoculum on the agar surface at the edge on the left hand side of the plate and streak the inoculum from side to side in parallel lines across the surface of the area.
6. Re-flame and cool the wire loop and turn the Petri dish at 90 degree. Make another group of parallel streaks perpendicular the first; re-flame the loop, allow to cool and make a third group of streak perpendicular to the second group.
7. Replace the lid of the Petri dish and incubate the plate in an inverted position at 25 degree Celsius for 48 to 72 hours.
8. Examine the plates for growth of bacterial colonies.
9. Flame a wire loop to red hot on a spirit lamp flame and cool it by dipping in a fresh agar plate.
10. Touch the tip of the loop to the surface of a selected discrete colony.
11. Lift the lid of the agar plate at 45 degree Celsius and inoculate by making parallel streaks on the agar surface.

Result

A divergent growth will be seen where the initial streak was made but the growth is less dense away from the streaks. Discrete colonies are formed on the third group of streaks. Any colony not growing on the streak marks is regarded as a contaminant.

EXPERIMENT 2: OBSERVATION OF SPOILAGE OF FERMENTED RED HOT CHILI PEPPER MASH

Hot pepper sauce is made from red hot chili peppers that is fermented for weeks to years depending on the processor. Food fermentation goes through several modifications that include physical, chemical and microbiological changes that affect

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the quality and safety of product. In the majority of fermentation processes of food products the prevailing microbial groups are Lactic Acid Bacteria (LAB) and/or yeast, the relative population of which defines the characteristics of the final product. When LAB grow during fermentation of food products, lactic acid fermentation is flavoured, rendering a more acidic product with a lower pH, which is highly desirable. The opposite happens when yeast or other spoilage microorganisms become the dominant group.

During the fermentation of the hot pepper sauce it is considered that LAB comes from the hot red chili peppers naturally and also from the fermenting barrel. However, these cultures exhibit diverse metabolic activities, which vary even among strains, including differences in growth rates, adaptation to a particular substrate, antimicrobial properties, flavour, aroma and quality attributes and competitive growth behaviour in mixed cultures.

The initial microorganisms in hot sauce fermentation are probably the same as in other agricultural products harvested under similar conditions of soil and weather. Besides the microorganisms found in the plant and soil where the peppers are grown, the peppers can contaminate from dust, fecal contamination from birds, rodents, insects, and from other sources during transportation, and processing of the red hot pepper mash. Researchers found that *Bacillus* is the most common isolate in spoiled hot red pepper sauce. Other microorganism included *Streptococcus*, *Leuconostoc*, and *Micrococcus* species among bacteria, and *Saccharomyces*, *Candida*, and *Rhodotorula* species among yeast. The most common fungi reported being present on red peppers were *Aspergillus*, *Penicillium*, and *Rhizopus*.

The fermentation of red hot chili mash results in approximately 7% of the barrels having spoiled pepper mash that have changes in colour, texture, and aroma. These changes include black or white pigmentation on the top or alongside the walls of the barrels, slime formation and an unpleasant aroma.

Methodology

Isolation and Identification of Spoilage Bacteria in Fermented Red Hot Chili Pepper Mash

In a general survey conducted, the barrels of red hot chili pepper mash were opened after two years of fermentation, it was observed that spoilage of mash on the top layer and walls of barrels. Spoiled mash had rotten aroma, black colour, and slime layer. Six samples of spoiled pepper mash were collected from wooden barrels and transported samples to the Food Safety Laboratory in an ice chest to analyse samples within 24 hours. Streak plate method was used to isolate colonies on Brain Hearth Infusion Agar (BHI), MacConkey Agar (MAC), and Lactobacilli MRS Agar (MRS). This process was repeated until isolated colonies grew in each plate. Once isolated, microorganisms were classified using Gram stain and

doing direct observation under the microscope. API® 20 STREP biochemical test identification system was used according to the manufacturer's instructions. Final identification of the microorganisms were obtained by calling APIweb™ database service provided by manufacturer.

Inoculation of Fresh Red Hot Chili Pepper Mash with Spoilage Isolates

Fresh pepper mash was inoculated under controlled conditions with isolated bacteria from spoiled mash, and observed for microbiological changes and spoilage during 120 days. This was achieved by inoculating each strain into normal fresh mash.

Results

Isolation and Identification of Spoilage Bacteria in Fermented Red Hot Chili Pepper Mash

From the microbiological analysis of spoiled red hot chili pepper mash *Bacillus firmus*, *Bacillus pumilus*, *Enterococcus avium*, *Brevibacillus laterosporus*, and *Aerococcus viridans* were isolated and identified. *Bacillus firmus* is a Gram-positive, rod-shaped bacterium. Colonies of *B. firmus* in Brain Heart Infusion (BHI) agar had a white translucent pigmentation, circular in shape, with undulated margins, and pulvinate elevation. When manipulated with a needle, the colonies were viscous (Refer Below Figure 1).



Fig. 1 *Bacillus firmus Isolated form Spoiled Red Hot Chili Pepper Mash*

Bacillus pumilus (*B. pumilus*) is a Gram-positive, aerobic, spore-forming, rod-shaped bacterium commonly isolated in soil, plants and environmental surfaces. Colonies of *B. pumilus* in BHI agar had a white pigmentation, surface of colonies was smooth and opaque, were punctiform with entire margins, and had a convex

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elevation. When manipulated with a needle, the colonies were butyrous (Refer Below Figure 2).



Fig. 2 *Bacillus pumilus Isolated form Spoiled Red Hot Chili Pepper Mash*

Enterococcus avium (*E. avium*) is a Gram-positive, cocci-shape bacterium. Enterococci are facultative anaerobic organisms that can survive and grow in many environments. Colonies of *E. avium* in BHI agar has a white pigmentation, surface was smooth and opaque, circular in shape with entire margin, and convex elevation. When manipulated with a needle, the colonies were butyrous (Refer Below Figure 3).



Fig. 3 *Enterococcus avium Isolated form Spoiled Red Hot Chili Pepper Mash*

Brevibacillus laterosporus (*B. laterosporus*) is a Gram-positive, rod shape bacterium. Colonies of *B. laterosporus* in BHI agar had a dull white translucent pigmentation, with a smooth surface, circular in shape with undulated margin and

raised elevation. When manipulated with a needle, the colonies were butyrous (Refer Below Figure 4).

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Fig. 4 *Brevibacillus laterosporus Isolated form Spoiled Red Hot Chili Pepper Mash*

Aerococcus viridans (*A. viridans*) is a Gram-positive cocci-shape bacterium. Colonies of *A. viridans* in BHI agar had a white opaque pigmentation, with smooth surface, circular in shape with entire margin, and convex elevation. When manipulated with a needle, the colonies were butyrous (Refer Below Figure 5).



Fig. 5 *Aerococcus viridans Isolated form Spoiled Red Hot Chili Pepper Mash*

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EXPERIMENT 3: PURE CULTURE AND PRESERVATION OF BACTERIA

A pure culture is a culture that contains only one kind of a microorganism as contrary to the mixed culture that contains more than one kind of microorganism. Pure culture, in microbiology is a laboratory culture that contains a single species of organism. It is usually derived from a mixed culture (one containing many species) by transferring a small sample into new, sterile growth medium in such a manner as to disperse the individual cells across the medium surface or by thinning the sample many fold before inoculating the new medium. Both methods separate the individual cells so that, when they multiply, each will form a discrete colony, which may then be used to inoculate more medium, with the assurance that only one type of organism will be present. Isolation of a pure culture may be enhanced by providing a mixed inoculum with a medium favouring the growth of one organism to the exclusion of others.

Pure cultures are essential in the study of the following aspects of microorganisms:

- Colony characteristics
- Biochemical and DNA-based identification
- Morphology
- Staining reactions

Obtaining Pure Culture of Microorganisms

The following points highlight the top six methods used for obtaining pure culture of microorganisms:

1. Streak Plate Method
2. Pour Plate Method
3. Spread Plate Method
4. Serial Dilution Method
5. Single Cell Isolation Methods
6. Enrichment Culture Method

1. Streak Plate Method

Streak Plate Method is commonly used to isolate pure cultures of bacteria. A small amount of mixed culture is placed on the tip of an inoculation loop/needle and is streaked across the surface of the agar medium. The successive streaks ‘thin out’ the inoculum sufficiently and the microorganisms are separated from each other.

It is usually advisable to streak out a second plate by the same loop/needle without reinoculation. These plates are incubated to allow the growth of colonies. The key principle of this method is that, by streaking, a dilution gradient is

established across the face of the Petri dish as bacterial cells are deposited on the agar surface. As this dilution gradient, confluent growth does not occur on that part of the medium where few bacterial cells are deposited.

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Apparently, each colony is the progeny of a single microbial cell thus representing a clone of pure culture. Such isolated colonies are picked up separately using sterile inoculating loop/needle and re-streaked onto fresh media to ensure purity.

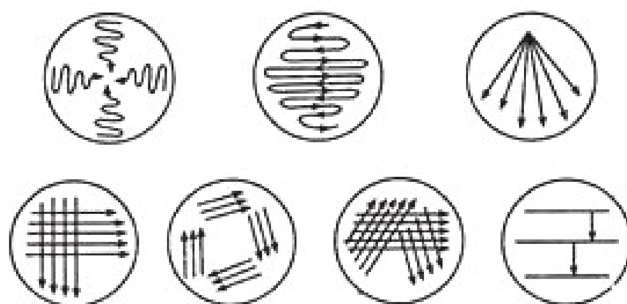




Fig. 1 *Different Methods of Streaking*

2. Pour Plate Method

The Pour Plate Method involves plating of diluted samples that is mixed with melted agar medium. The main principle is to dilute the inoculum in successive tubes containing liquefied agar medium so to allow a thorough distribution of bacterial cells within the medium.

Here, the mixed culture of bacteria is diluted directly in tubes containing melted agar medium maintained in the liquid state at a temperature of 42-45°C (agar solidifies below 42°C). The bacteria and the melted medium are mixed well.

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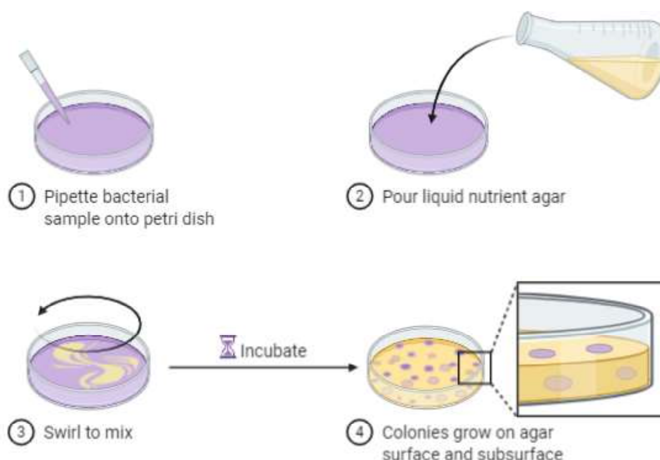


Fig. 2 *Pour Plate Method*

The contents of each tube are poured into separate Petri dish, allowed to solidify, and then incubated. When bacterial colonies develop, one finds that isolated colonies develop both within the agar medium (subsurface colonies) and on the medium (surface colonies). These isolated colonies are then picked up by inoculation loop and streaked onto another Petri dish to insure purity.

Disadvantages of Pour Plate Method

Following are the disadvantages of Pour Plate Method:

- The picking up of subsurface colonies needs digging them out of the agar medium thus interfering with other colonies.
- The microbes being isolated must be able to withstand temporary exposure to the 42-45° C temperature of the liquid agar medium; therefore this technique is not suitable for the isolation of psychrophilic microorganisms.

However, the pour plate method, in addition to its use in isolating pure cultures, is also used for determining the number of viable bacterial cells present in a culture.

3. Spread Plate Method

In Spread Plate Method, the mixed culture or microorganisms is diluted in a series of tubes containing sterile liquid, usually, water or physiological saline. A drop of so diluted liquid from each tube is placed on the centre of an agar plate and spread evenly over the surface by means of a sterilized bent-glass-rod. The medium is now incubated. When the colonies develop on the agar medium plates, it is found that there are some plates in which well-isolated colonies grow. This happens as a result of separation of individual microorganisms by spreading over the drop of diluted liquid on the medium of the plate.

The isolated colonies are picked up and transferred onto fresh medium to ensure purity. In contrast to pour plate method, only surface colonies develop in this method and the microorganisms are not required to withstand the temperature of the melted agar medium.

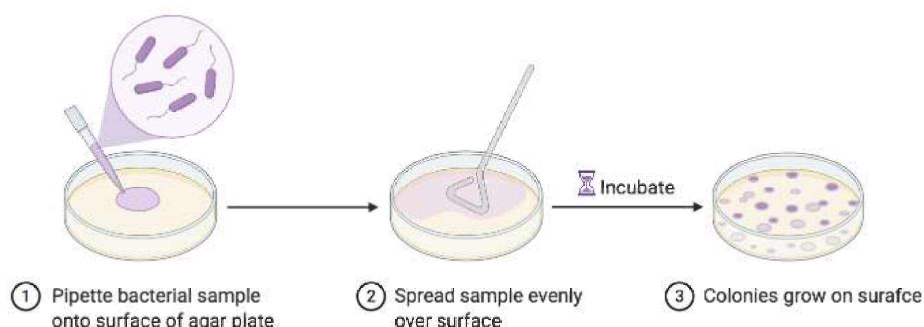


Fig. 3 *Spread Plate Method*

4. Serial Dilution Method

Serial Dilution Method is commonly used to obtain pure cultures of those microorganisms that have not yet been successfully cultivated on solid media and grow only in liquid media.

A microorganism that predominates in a mixed culture can be isolated in pure form by a series of dilutions. The inoculum is subjected to serial dilution in a sterile liquid medium, and a large number of tubes of sterile liquid medium are inoculated with aliquots of each successive dilution. The aim of this dilution is to inoculate a series of tubes with a microbial suspension so dilute that there are some tubes showing growth of only one individual microbe. For convenience, suppose we have a culture containing 10 ml of liquid medium, containing 1,000 microorganisms, i.e., 100 microorganisms/ml of the liquid medium.

If we take out 1 ml of this medium and mix it with 9 ml of fresh sterile liquid medium, we would then have 100 microorganisms in 10 ml or 10 microorganisms/ml. If we add 1 ml of this suspension to another 9 ml. of fresh sterile liquid medium, each ml would now contain a single microorganism.

If this tube shows any microbial growth, there is a very high probability that this growth has resulted from the introduction of a single microorganism in the medium and represents the pure culture of that microorganism.

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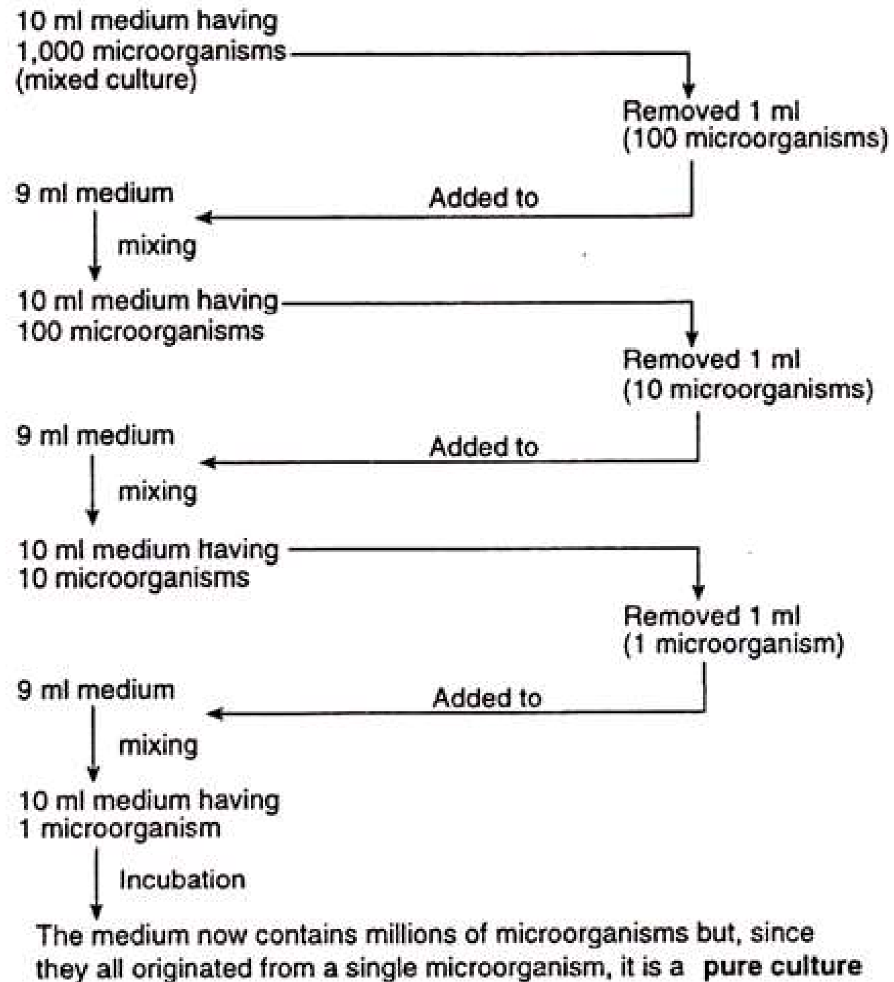


Fig. 4 *Serial Dilution Method*

5. Single Cell Isolation Method

In Single Cell Isolation Method an individual cell of the required kind is picked out by this method from the mixed culture and is permitted to grow.

Methods of Single Cell Isolation

The following two methods are in use:

- Capillary Pipette Method
- Micromanipulator Method

A. Capillary Pipette Method

In Capillary Pipette Method several small drops of a suitably diluted culture medium are put on a sterile glass-coverslip by a sterile pipette drawn to a capillary. One then examines each drop under the microscope until one finds such a drop, which contains only one microorganism. This drop is removed with a sterile capillary pipette to fresh medium. The individual microorganism present in the drop starts multiplying to yield a pure culture.

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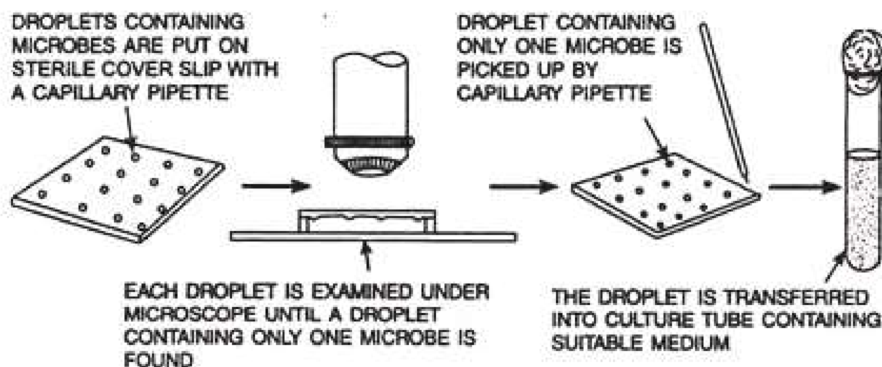


Fig. 5 *Capillary Pipette Method for Obtaining a Single Microbial Cell*

B. Micromanipulator Method

Micromanipulators pick out a single cell from a mixed culture. This instrument is used in conjunction with a microscope to pick a single cell (particularly bacterial cell) from a hanging drop preparation. The micro-manipulator has micrometre adjustments by means of which its micropipette can be moved right and left, forward, and backward, and up and down. A series of hanging drops of a diluted culture are placed on a special sterile coverslip by a micropipette. Now a hanging drop is searched, which contains only a single microorganism cell.

This cell is drawn into the micropipette by gentle suction and then transferred to a large drop of sterile medium on another sterile coverslip. When the number of cells increases in that drop as a result of multiplication, the drop is transferred to a culture tube having suitable medium. This yields a pure culture of the required microorganism.

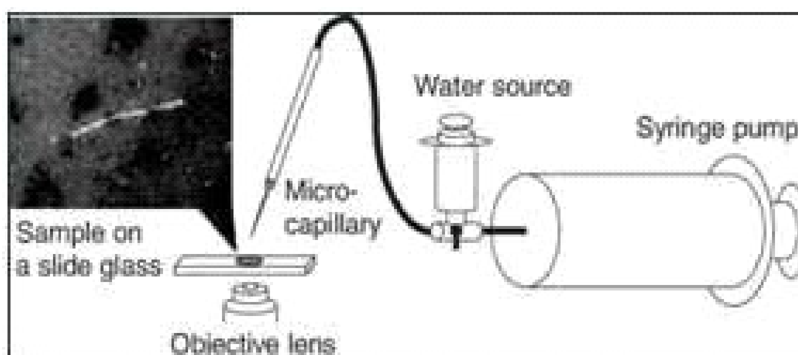


Fig. 6 *Micromanipulator Method for Pure Culture*

The advantages of this method are that one can be reasonably sure that the cultures come from a single cell and one can obtain strains with in the species. The disadvantages are that the equipment is expensive, its manipulation is very tedious, and it requires a skilled operator. This is the reason why this method is reserved for use in highly specialised studies.

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6. Enrichment Culture Method

Generally, Enrichment Culture Method is used to isolate those microorganisms, which are present in relatively small numbers or that have slow growth rates compared to the other species present in the mixed culture.

The enrichment culture strategy provides a specially designed cultural environment by incorporating a specific nutrient in the medium and by modifying the physical conditions of the incubation. The medium of known composition and, specific condition of incubation favours the growth of desired microorganisms but, is unsuitable for the growth of other types of microorganisms.

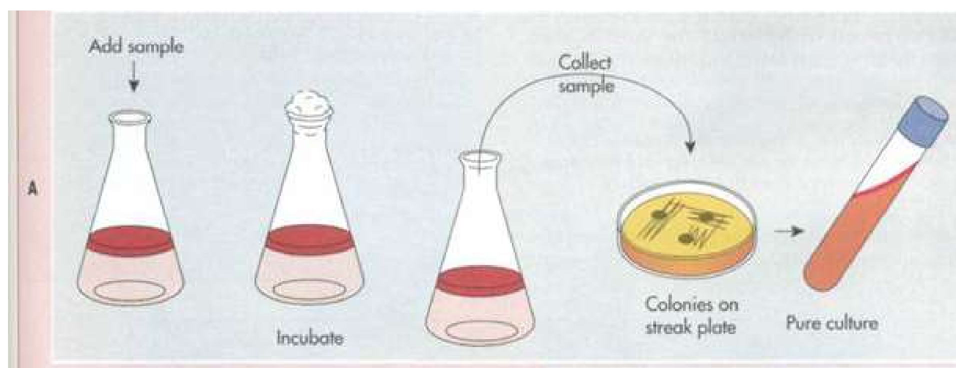


Fig. *7 Enrichment of Specific Bacteria*

Maintenance and Preservation of Pure Cultures

The following points highlight the top four methods used for maintenance and preservation of pure cultures:

1. Refrigeration
2. Paraffin Method
3. Cryopreservation
4. Lyophilisation or Freeze-Drying

1. Refrigeration

Pure cultures can be successfully stored at 0-4°C either in refrigerators or in cold rooms. This method is applied for short duration (2-3 weeks for bacteria and 3-4 months for fungi) because the metabolic activities of the microorganisms are greatly slowed down but not stopped. Thus, their growth continues slowly, nutrients are utilized and waste products released in medium. This results in, finally, the death of the microbes after sometime.

2. Paraffin Method

Paraffin Method is a simple and most economical method of maintaining pure cultures of bacteria and fungi. In this method, sterile liquid paraffin in poured over the slant (slope) of culture and stored upright at room temperature. The layer of paraffin ensures anaerobic conditions and prevents dehydration of the medium.

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This condition helps microorganisms or pure culture to remain in a dormant state and, therefore, the culture is preserved for several years.

The advantage of this method is that we can remove some of the growth under the oil with a transfer needle, inoculate a fresh medium, and still preserve the original culture. The simplicity of the method makes it attractive, but changes in the characteristics of a strain can still occur.

3. Cryopreservation

Cryopreservation, i.e., freezing in liquid Nitrogen at -196°C or in the gas phase above the liquid Nitrogen at -150°C helps the survival of pure cultures for long storage times.

In this method, the microorganisms of culture are rapidly frozen in liquid Nitrogen at -196°C in the presence of stabilizing agents, such as glycerol or DiMethyl SulfOxide (DMSO) that prevent cell damage due to the formation of ice crystals and promote cell survival. This liquid Nitrogen method has been successful with many species that cannot be preserved by lyophilisation and most species can remain viable under these conditions for 10 to 30 years without undergoing a change in their characteristics, however, this method is expensive.

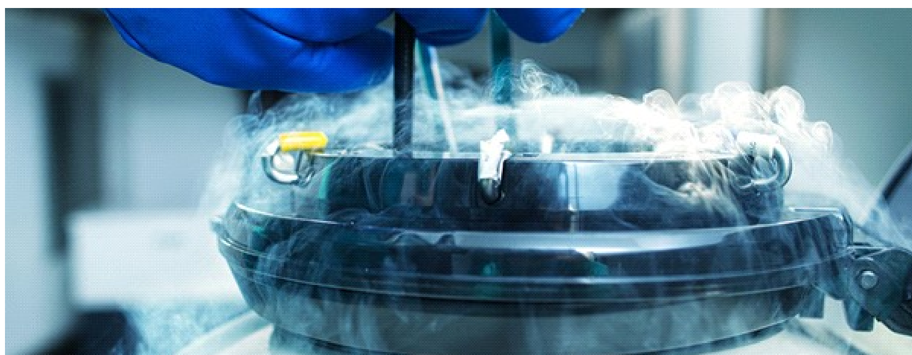


Fig. 8 *Cryopreservation*

4. Lyophilisation or Freeze-Drying

Lyophilisation or Freeze-Drying is a process where water and other solvents are removed from a frozen product via sublimation. Sublimation occurs when a frozen liquid goes directly to a gaseous state without entering a liquid phase.

It is recommended to use slow rates of cooling, as this will result in the formation of vertical ice crystal structures, thus allowing for more efficient water sublimation from the frozen product. Freeze-dried products are hygroscopic and must be protected from moisture during storage. Under these conditions, the microbial cells are dehydrated and their metabolic activities are stopped; as a result, the microbes go into a dormant state and retain viability for years. Lyophilized or freeze-dried pure cultures are then sealed and stored in the dark at 4°C in refrigerators.

Freeze-drying method is the most frequently used technique by culture collection centres. Many species of bacteria preserved by this method have remained viable and unchanged in their characteristics for more than 30 years.

Advantages of Lyophilisation

Following are the advantages of Lyophilisation:

- Only minimal storage space is required; hundreds of lyophilized cultures can be stored in a small area.
- Small vials can be sent conveniently through the mail to other microbiology laboratories when packaged in a special sealed mailing container.
- Lyophilized cultures can be revived by opening the vials, adding the liquid medium, and transferring the rehydrated culture to a suitable growth medium.

EXPERIMENT 4: GRAM STAINING

Gram staining is a common technique used to differentiate two large groups of bacteria based on their different cell wall constituents. The Gram stain procedure distinguishes between Gram-positive and Gram-negative groups by colouring these cells red or violet. Gram-positive bacteria stain violet due to the presence of a thick layer of peptidoglycan in their cell walls, which retains the crystal violet these cells are stained with. Alternatively, Gram-negative bacteria stain red, which is attributed to a thinner peptidoglycan wall, which does not retain the crystal violet during the decolouring process.

Process of Gram Staining

Gram staining involves three processes: staining with a water-soluble dye called crystal violet, decolourization, and counterstaining, usually with safranin. Due to differences in the thickness of a peptidoglycan layer in the cell membrane between Gram-positive and Gram-negative bacteria, Gram-positive bacteria (with a thicker peptidoglycan layer) retain crystal violet stain during the decolourization process, while Gram-negative bacteria lose the crystal violet stain and are instead stained by the safranin in the final staining process. The process involves three steps:

1. Cells are stained with crystal violet dye. Next, a Gram's Iodine solution (Iodine and Potassium Iodide) is added to form a complex between the crystal violet and Iodine. This complex is a larger molecule than the original crystal violet stain and iodine and is insoluble in water.
2. A decolourizer, such as Ethyl Alcohol or Acetone is added to the sample, which dehydrates the peptidoglycan layer, shrinking and tightening it. The large crystal violet-iodine complex is not able to penetrate this tightened peptidoglycan layer, and is thus trapped in the cell in Gram-positive bacteria. Conversely, the outer membrane of Gram-negative bacteria is degraded and the thinner peptidoglycan layer of Gram-negative cells is unable to retain the crystal violet-iodine complex and the colour is lost.

3. A counterstain, such as the weakly water soluble safranin, is added to the sample, staining it red. Since, the safranin is lighter than crystal violet, it does not disrupt the purple coloration in Gram-positive cells. However, the decolorized Gram-negative cells are stained red.

Specimen Collection

Various clinical specimens can be used to perform Gram staining. Some of the commonly used specimens are sputum, blood, cerebrospinal fluid, ascitic fluid, synovial fluid, pleural fluid, and urine, etc. Swabs from nostrils, throat, rectum, wound, and cervix, etc. can also be used. The collection of specimens should always be in sterile containers.

Aim

To understand the staining procedures used in the study of bacteria.

Materials Required

- Bunsen Burner
- Alcohol-Cleaned Microscope Slide
- Slide Rack
- Microscope
- Inoculating Loop
- Bibulous Paper
- Microscope
- Lens Paper and Lens Cleaner
- Immersion Oil
- Distilled Water
- 18 to 24 Hour Cultures of Organisms

Reagents Needed for Gram Staining

- Crystal Violet (Primary Stain)
- Grams Iodine Solution (The Mordant)
- Ethyl Alcohol (The Decolourizer)
- Safranin (Secondary Stain)
- Water

Procedure

1. Preparation of a Slide Smear

- Inoculation loop is used to transfer a drop of suspended culture to the microscope slide.
- If a Petri dish or a slant culture tube has the colony, a drop or a few loopful of water is added to facilitate a minimal amount of colony transfer to the examination slide.

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- A minimal amount of culture is required. If culture can be detected visually on an inoculation loop, it indicates the collection of too much culture.
- Culture is spread with an inoculation loop to an even thin film over a circle of 15mm in diameter. A typical slide can contain up to 4 small smears if examining more than one culture.
- The slide can be either air-dried or dried with the help of heat over a gentle flame. The slide should be moved circularly over the flame to prevent overheating or forming of ring patterns in the slide. The heat helps the cell adhesion to the glass slide and prevents the significant loss of culture during rinsing.

2. Labelling of the Slides

Draw a circle on the underside of the slide using a glassware-marking pen to clearly designate the area in which you will prepare the smear. You may also label the slide with the initials of the name of the organism on the edge of the slide. The label should not be in contact with the staining reagents.

3. Heat Fixing

Heat fixing kills the bacteria in the smear, firmly adheres the smear to the slide, and allows the sample to more readily take up stains.

- Allow the smear to air dry.
- After the smear has air-dried, hold the slide at one end and pass the entire slide through the flame of a Bunsen burner two to three times with the smear-side up.

Now the smear is ready to be stained.

Note: Prevent the overheating of slide as proteins in the specimen can coagulate causing cellular morphology to appear distorted.

4. Gram Staining

- Place slide with heat fixed smear on staining tray.
- Gently flood smear with crystal violet and let stand for 1 minute.
- Tilt the slide slightly and gently rinse with tap water or distilled water using a wash bottle.
- Gently flood the smear with Grams Iodine and let stand for 1 minute. This step is known as 'Fixing the Dye'. Iodine solution is poured off, and the slide is rinsed with running water. Excess water from the surface is shaken off.
- Tilt the slide slightly and gently rinse with tap water or distilled water using a wash bottle. The smear will appear as a purple circle on the slide.
- Decolourize using 95% Ethyl Alcohol or Acetone. Tilt the slide slightly and apply the Alcohol drop by drop for 5 to 10 seconds until the Alcohol runs almost clear. Be careful not to over-decolorize. To prevent excess

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decolourization in the Gram-positive cells, stop adding decolourizer as soon as the solvent is not coloured as it flows over the slide. Decolourizers are often the mixed solvent of Ethyl Alcohol and Acetone. This step is known as 'Solvent Treatment'.

- Immediately rinse with water.
- Gently flood with safranin to counter-stain and let stand for 45 seconds.
- Tilt the slide slightly and gently rinse with tap water or distilled water using a wash bottle.
- Blot dry the slide with bibulous paper.
- View the smear using a light-microscope under oil-immersion.

5. Microscopic Examination of Slide

- The slide should undergo an examination under a microscope under oil immersion.
- The initial slide examination should use the X40 objective to evaluate the smear distribution, and then they should be examined using the X100 oil immersion objective.
- All areas of the slide require an initial examination. Areas that are only one cell thick should be examined. Thick areas in slides often give variable and incorrect results.
- White blood cells and macrophages stain Gram-negative.
- Squamous epithelial cells stain Gram-positive.

Various modifications of Gram staining are used, such as Atkin Gram stain, and Burke Gram stain, etc.

Indications: Gram staining is indicated whenever a bacterial infection is suspected for easy and early diagnosis.

Examples of Gram-Negative and Gram-Positive Bacteria

Gram-Negative Bacteria

- *Bordetella pertussis*, the causative agent of whooping cough.
- *Salmonella typhi*, the causative agent of typhoid.
- *Vibrio cholera*, the causative agent of cholera.
- *Escherichia coli*, the normally benign, ubiquitous, gut-dwelling bacteria.

Gram-Positive Bacteria

- *Staphylococci*, such as *Staphylococcus epidermidis* and *Staphylococcus aureus* which is a common cause of boils.
- *Streptococci*, such as the many species of oral streptococci, *Streptococcus pyogenes* which causes many a sore throat and scarlet fever and *Streptococcus pneumoniae* which causes lobar pneumonia.

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- *Clostridia,* such as *Clostridium tetani*, the causative agent of tetanus (lockjaw).
- *Actinomyces*, such as *Actinomyces odontolyticus* which is found in mouth.
- Species of the genus Bacillus, such as *Bacillus subtilis* which are common microbes living in soil.

Generally cocci are Gram-positive but there are exceptions. The most significant from a clinical point of view is the gonococcus, *Neisseria gonorrhoea* which typically appears as a Gram-negative diplococcus looking very much like a pair of kidney bean.

Complications

The interpretation of slides can be difficult if the microscopic smear is thick and clumped. Decolourization time should have very close monitoring to avoid under-decolourization or over-decolourization. Thicker smears require longer decolorizing time. Similarly, cultures should undergo evaluation while they are still fresh. Old cultures tend to lose the peptidoglycan cell walls, which predisposes Gram-positive cells to be Gram-negative or Gram variable. Gram stain is not useful for organisms without a cell wall like *Mycoplasma* species, and for smaller bacteria like *Chlamydia* and *Rickettsia* species.

Gram stain may not show the adequate in the following condition:

- Use of antibiotics before collecting a specimen.
- Inappropriate age of culture: too young or too old.
- Fixing the smear before it is dry.
- The smear is too thick.
- Low concentration of crystal violet.
- Excessive heat fixation.
- Excessive washing between steps.
- Insufficient exposure to Iodine.
- Prolonged decolourization.
- Excessive counterstaining.
- Lack of experience in preparing the slide, and reviewing the slide.

Sometimes results of Gram-stain may not match the final results of cultures and could potentially lead to inappropriate use of antibiotics.

EXPERIMENT 5: MOTILITY TEST

Introduction

Motility, is considered as an important taxonomic tool, is the capability of an organism through flagella or fibrils. The flagella, present in motile bacteria, extend outward from the plasma membrane and cell wall; there can be either single

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flagellum or multiple flagella. Motility is an important characteristic feature of bacteria. The presence of flagella occurs primarily in bacilli group of bacteria.

Objectives

1. To understand the motility of bacterium.
2. To distinguish between motile and non-motile bacteria.

Principle

Motility by bacterium is mostly demonstrated in a semi solid agar medium where they 'Swarm' and give a diffused spread in their growth. This feature is easily visualized by the naked eyes. The most commonly used medium is SIM medium (Sulphide Indole Motility medium) that is utilized for testing three different parameters, Sulphur Reduction, Indole Production and Motility. Since SIM medium has a very soft consistency, it permits motile bacteria to travel readily through them. This results in the cloudiness of the medium. The inoculum is introduced into the centre of the medium where a diffused zone of bacterial growth extending out, is observed. The non-motile bacteria will not show diffused growth and can be seen only in the soft agar tube and the area where they are inoculated.

Materials Required

- Media: SIM Medium
- Pancreatic Digest of Casein 20.0 g
- Peptic Digest of Animal Tissue 6.1g
- Agar 3.5g
- Ferric Ammonium Sulphate - $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ 0.2g
- Sodium Thiosulfate Crystals - $Na_2S_2O_3 \cdot 5H_2O$ 0.2g
- pH 7.3 ± 0.2 at 25°C

Method

- Touch a straight needle to a colony of a young (18- to 24-hour) culture growing on agar medium.
- Stab once to a depth of only 1/3 to ½ inch in the middle of the tube. Be sure to keep the needle in the same line it entered as it is removed from the medium.
- Incubate at 35°-37°C and examine daily for up to 7 days.
- Observe for a diffuse zone of growth flaring out from the line of inoculation.

Results

Positive (Motile Bacteria): Diffused growth throughout the medium resulting in cloudiness.

Negative (Non-Motile Bacteria): Growth localized to stab-line.

Uses

- It is used for the differentiation of microorganisms on the basis of motility in a laboratory setting.
- It is performed to assign taxonomic classification to organisms.
- Motility tests are important in characterization of pathogens.
- The tests are often employed in identification protocols in the family *Enterobacteriaceae*
- Motility test is also used for the species differentiation of Gram-positive cocci, *Enterococci*. *Enterococcus faecium* and *Enterococcus faecalis* are non-motile, whereas *Enterococcus gallinarum* and *Enterococcus casseliflavus, Enterococcus flavescens* generally are motile.

Limitations

- Some organisms will not display sufficient growth in this medium to make an accurate determination, and additional follow-up testing is required.
- It is recommended that biochemical, immunological, molecular, or mass spectrometry testing be performed on colonies from pure culture for complete identification.
- False-negative reactions may occur if bacterial flagella are damaged due to heating, shaking, or other trauma. Such environmental shock will render the organism non-motile.
- Organisms which are weakly motile may result in false-negative reactions.
- When inoculating semi-solid media, it is important that the inoculating needle be removed along the exact same line used to inoculate the medium. A fanning motion may result in growth along the stab line that may result in false-positive interpretation.

EXPERIMENT 6: HYDROLYSIS OF STARCH, GELATIN AND PROTEIN

1. HYDROLYSIS OF GELATIN

Gelatin hydrolysis test is a biochemical test performed as a presumptive test for the identification of *Staphylococcus*, *Enterococcus*, and other Gram-positive bacilli. Gelatin hydrolysis test is also termed as the Gelatin liquefaction test as it involves the liquefaction of gelatin in the presence of the gelatinase enzyme. Gelatinase is an important enzyme in various pathogenic organisms as it is produced extracellularly, which hydrolyses gelatin which is derived from the collagen found in the connective tissues of vertebrates. These enzymes might work as a virulence factor that dissolves the connective tissues of the host's cells which aids in the invasive infections. Gelatin is a protein that liquefies in the presence of gelatinase enzyme as the enzyme breaks down the complex structure of gelatin into monomeric

amino acids. The test has been used for years as a presumptive test for the identification of a wide variety of organisms like *Serratia, Pseudomonas*, *Flavobacterium*, and *Clostridium*.

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Aim

To study the ability of microorganisms to hydrolyze gelatin with the proteolytic enzyme gelatinase.

Objectives of Gelatin Hydrolysis Test

- To test an organism's ability to liquefy gelatin by the production of gelatinase enzyme.
- To differentiate organisms into different groups based on their ability to hydrolyze gelatin.

Principle of Gelatin Hydrolysis Test

The ability of microorganisms to hydrolyze gelatin is commonly taken as evidence that the organism can hydrolyze protein in general. Microorganisms vary from species to species with regard to their ability to hydrolyze protein in some species. Gelatin is a protein obtained by the hydrolysis of a collagen compound of the connective tissues of animals. It is convenient as a substrate for proteolytic enzymes in microorganisms. Gelatin is used as the media from the experiment, which is liquid at room temperature and solidifies at –4°C. If the gelatin has been hydrolyzed by the action of organism the media will remain liquid.

Materials Required

- Nutrient Gelatin Media
- Test Organism
- Test Tubes
- Inoculation Loop
- Media: 1% Gelatin Agar
- Mercuric Chloride ($HgCl_2$)
- Culture: 24 Hours Culture of *Bacillus* spp and *coli*

Procedures

1.Gelatin Hydrolysis Test in Agar Plate

- Prepare agar media with 1% gelatin.
- Inoculate the gelatin agar plate with the given organism (*coli* and *Bacillus* in separate plate) using inoculating loop making single central streak in the plate.
- Incubate the plates at 37 °C for 24 to 48 hours.
- Flood the plates with $HgCl_2$ solution.
- Observe the plates after few minutes. A clear halo-zone around the inoculated area indicates gelatin hydrolysis; positive test.

2.Gelatin Hydrolysis Test in Tube

- Prepare gelatin media in test tube.
- Inoculate the gelatin tube with given organism by stabbing the media multiple time using inoculating wire.
- Incubate the tube at room temperature (25°C) for 24 to 48 hours.
- After incubation for 24 to 48 hours, place the tube in refrigerator for 30 minutes at 4° C.
- Observe the tube for gelatin hydrolysis. The hydrolyzed tube remain liquid even after freezing at 4°C

Different Steps

- Media is prepared according to the above given composition.
- It is sterilized at 121°C for 15 minutes at 15 lb/square inch and poured into presterilized tubes.
- Tubes are allowed to cool and then inoculated with test organisms with 1 inoculated tube used as a control.
- Tubes are incubated for 24 hours and observed for liquefaction of gelatin, after keeping them in ice for half an hour.

Result

Gelatin is an incomplete protein, lacking many amino acids, such as tryptophan. When collagen is heated and hydrolyzed, denatured protein gelatin is obtained. Collagen accounts for 90% to 95% of organic matter in the cell. It is the most important protein, rich in amino acids. Microorganisms like bacteria can use gelatin only if they are supplemented with other proteins. Bacteria produce the gelatin-hydrolysis enzyme gelatinase. Since gelatine is a good solidifying agent at low temperatures, its property of solidification can be used to distinguish between gelatin- hydrolysis and non- hydrolysis agents. Most of the Enterobacteriaceae are gelatin-hydrolysis-test–negative. Bacteria like *Vibrio*, *Bacillus*, and *Pseudomonas* are gelatin-positive.

Microorganisms Tested

- Gram-negative rods that require gelatin for identification, especially for the separation of the fluorescent Pseudomonas: *Pseudomonas putida* (negative) from *Pseudomonas fluorescens* (positive).
- Gram-positive rods as needed for identification to the species level.
- Gelatin hydrolysis bacteria: *Serratia*, *Proteus*, *Staphylococcus aureus*, *Bacillus spp.*, *Clostridium spp.*, *Pseudomonas.*
- Gelatin non-hydrolysis bacteria: Members of *Enterobacteriaceae* family, *Staphylococcus epidermidis*

Uses of Gelatin Hydrolysis Test

- This test is used to determine the ability of an organism that produce gelatinases.
- This test is helpful in identifying and differentiating species of *Serratia*, *Proteus*, *Bacillus*, *Clostridium*, *Pseudomonas* and *Flavobacterium*.
- This test differentiates pathogenic *Staphylococcus aureus* which is gelatinase-positive from non-pathogenic epidermidis which is gelatinase negative.
- This test can be used to differentiate *Serratia* and *Proteus* species which are gelatin positive from other members of *Enterobacteriaceae* family.
- *Bacillus anthracis*, *B. cereus* and several other members of the genus are gelatinase-positive, as are *Clostridium tetan*i and *Perfringens*.

Limitation

- Gelatinase usually acts at the surface of the medium. Shaking the tube while it is warm may result in false-negative interpretation.
- Gelatin may vary in gelling ability. Therefore, incubate an uninoculated control with the test. The control must be refrigerated along with the test, prior to reading.

Observation Table

Type of Biochemical Test	Observation	Conclusion (Positive or Negative)

2. HYDROLYSIS OF STARCH

Starch is an energy-storage carbohydrate in plants. It is composed of two constituents: amylose and amylopectin, made of repeating alpha-D-glucose linked by glycosidic bonds. Starch as such cannot be transported into the cell for energy production, because of its high molecular weight. Bacteria that produce extracellular enzymes break down starch into glucose subunits. These are transported into the cell, where they are broken down by the endoenzymes in cell respiration.

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Starch is a polymer of glucose that is too large to be transported into bacterial cells. For bacteria to be able to use the glucose in starch as an energy source, the macromolecule must be broken down extracellularly into smaller monosaccharide and disaccharide subunits. Some bacteria secrete the extracellular enzyme (exoenzyme) amylase that breaks down starch into monosaccharides and disaccharides (glucose and maltose). These smaller molecules can then be transported inside the bacterial cell.



Starch agar is used to determine if bacteria can produce the amylase enzyme. Bacteria that secrete amylase will break down the starch in the medium surrounding the bacterial colony. After the plate is inoculated and incubated, iodine is poured on the plate. The combination of iodine and starch results in the formation of a dark blue-black colour. Regions of the agar where no starch is present (i.e., where starch has been broken down by amylase) will remain the colour of the iodine (light yellow or gold).

Objectives

To study the various parameters that affect the kinetics of alpha-amylase catalyzed hydrolysis of starch.

Introduction

Starchy substances constitute the major part of the human diet for most of the people in the world, as well as many other animals. They are synthesized naturally in a variety of plants. Some plant examples with high starch content are corn, potato, rice, sorghum, wheat, and cassava. It is no surprise that all of these are part of what we consume to derive carbohydrates. Similar to cellulose, starch molecules are glucose polymers linked together by the alpha-1, 4 and alpha-1,6 glycosidic bonds, as opposed to the beta-1,4 glycosidic bonds for cellulose. In order to make use of the carbon and energy stored in starch, the human digestive system, with the help of the enzyme amylases, must first break down the polymer to smaller assimilable sugars, which is eventually converted to the individual basic glucose units.

Because of the existence of two types of linkages, the alpha-1, 4 and the alpha-1, 6, different structures are possible for starch molecules. An unbranched, single chain polymer of 500 to 2000 glucose subunits with only the alpha-1, 4 glycosidic bonds is called amylose. On the other hand, the presence of alpha-1, 6 glycosidic linkages results in a branched glucose polymer called amylopectin. The degree of branching in amylopectin is approximately one per twenty-five glucose units in the unbranched segments. Another closely related compound functioning as the glucose storage in animal cells is called glycogen, which has one branching per 12 glucose units. The degree of branching and the side chain length vary from

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source to source, but in general the more the chains are branched, the more the starch is soluble.

Starch is generally insoluble in water at room temperature. Because of this, starch in nature is stored in cells as small granules which can be seen under a microscope. Starch granules are quite resistant to penetration by both water and hydrolytic enzymes due to the formation of hydrogen bonds within the same molecule and with other neighbouring molecules. However, these inter and intra-hydrogen bonds can become weak as the temperature of the suspension is raised. When an aqueous suspension of starch is heated, the hydrogen bonds weaken, water is absorbed, and the starch granules swell. This process is commonly called gelatinization because the solution formed has a gelatinous, highly viscous consistency. The same process has long been employed to thicken broth in food preparation.

Depending on the relative location of the bond under attack as counted from the end of the chain, the products of this digestive process are dextrin, maltotriose, maltose and glucose, etc. Dextrin are shorter, broken starch segments that form as the result of the random hydrolysis of internal glycosidic bonds. A molecule of maltotriose is formed if the third bond from the end of a starch molecule is cleaved; a molecule of maltose is formed if the point of attack is the second bond; a molecule of glucose results if the bond being cleaved is the terminal one; and so on. The initial step in random depolymerization split of large chains into various smaller sized segments. The breakdown of large particles drastically reduces the viscosity of gelatinized starch solution, resulting in a process called liquefaction because of the thinning of the solution. The final stages of depolymerization are mainly the formation of mono-, di-, and tri-saccharides. This process is called saccharification, due to the formation of saccharides.

Since, a wide variety of organisms, including humans, can digest starch, alpha-amylase is obviously widely synthesized in nature, as opposed to cellulase. For example, human saliva and pancreatic secretion contain a large amount of alpha-amylase for starch digestion. The specificity of the bond attacked by alpha-amylases depends on the sources of the enzymes. Currently, two major classes of alpha-amylases are commercially produced through microbial fermentation. Based on the points of attack in the glucose polymer chain, they can be classified into two categories, liquefying and saccharifying.

Because the bacterial alpha-amylase to be used in this experiment randomly attacks only the alpha-1, 4 bonds, it belongs to the liquefying category. The hydrolysis reaction catalyzed by this class of enzymes is usually carried out only to the extent that, for example the starch is rendered soluble enough to allow easy removal from starch-sized fabrics in the textile industry. The paper industry also uses liquefying amylases on the starch used in paper coating where breakage into the smallest glucose subunits is actually undesirable.

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On the other hand, the fungal alpha-amylase belongs to the saccharifying category and attacks the second linkage from the non-reducing terminals (i.e., C4 end) of the straight segment, resulting in the splitting off of two glucose units at a time. Thus, the product is a disaccharide called maltose. The bond breakage is thus more extensive in saccharifying enzymes than in liquefying enzymes. The starch chains are literally chopped into small bits and pieces. Finally, the amyloglucosidase (also called glucoamylase) component of an amylase preparation selectively attacks the last bond on the non-reducing terminals. Fungal amylase and amyloglucosidase can be used together to convert starch to simple sugars. The practical applications of this type of enzyme mixture include the production of corn syrup and the conversion of cereal mashes to sugars in brewing.

Thus, it is important to specify the source of enzymes when the actions and kinetics of the enzymes are compared. Four types of alpha-amylases from different sources will be employed in this experiment: three of microbial origin and one of human origin. The effects of temperature, pH, substrate concentration and inhibitor concentration on the kinetics of amylase catalyzed reactions will be studied. Finally, the action of the amylase preparations isolated from microbial sources are compared to that from human saliva.

List of Reagents and Equipment

Equipment

- Erlenmeyer Flasks
- Beakers
- Graduated Cylinder
- Pipets, 1ml and 10ml
- Test Tubes
- Temperature Bath
- Thermometer
- Balance
- Syringe
- Filter Holder and Filter Paper
- Spectrophotometer
- Brookfield Viscometer

Reagents

- Enzymes
  - o Bacterial amylase solution, 3000 SKB units/ml
  - o Fungal amylase powder, 40,000 SKB units/g. (Concentration of the fungal amylase solution to be used in class: 75g/l)

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  - Amyloglucosidase solution, 75 AG units/ml
  - Human salivary amylase
- Corn starch
- HCl Stopping Solution, 0.1N HCl
- Iodine Reagent Stock Solution (in aqueous solution)
  - Iodine: 5 g/l
  - KI: 50 g/l
- Potassium Phosphate Buffers
  - KH_2PO_4 (Monobasic Phosphate) (FW=136.1)
  - $K_2HPO_4 \cdot 3H_2O$ (Dibasic Phosphate) (FW=228.23)
- $CaCl_2 \cdot 2H_2O$, 0.1M solution
- Reagents for the analysis of reducing sugars

Procedures

1.Prepare a 20 g/l Starch Solution

- Mix 20 g of soluble potato starch in approx. 50 ml of cold water.
- While stirring, add the slurry to approx. 900 ml of gently boiling water in a large beaker.
- Mix well and cool the gelatinized starch solution to room temperature.
- Add more water to bring the total volume to 1 litter.
- Put a few drops of the starch solution on a glass plate. Add 1 drop of the iodine reagent and see that a deep blue colour is developed. The blue colour indicates the presence of starch in the solution.

2.Effect of the pH

- Prepare 0.1M pH buffer solutions ranging from pH 4.5 to pH 9 in increments of one pH unit. Before coming to the lab, review how to make a pH buffer solution and calculate the relative amounts of KH_2PO_4 (monobasic phosphate) and $K_2HPO_4 \cdot 3H_2O$ (dibasic phosphate) needed to make these phosphate buffer solutions.
- Add an equal volume of one of the above buffer solutions to 5.0ml of the 20g/l starch solution. The resulting solution should contain 10g/l of starch in a buffered environment.
- Start the enzymatic digestion process by adding 0.5 ml of the bacterial amylase solution; shake and mix.
- Let the hydrolysis reaction proceed for exactly 10 minutes at 25ºC.
- Add 0.5 ml of the reacted starch solution to 5ml of the HCl stopping solution (0.1N).

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- Add 0.5 ml of the above mixture to 5ml iodine solution to develop colour. Shake and mix. The solution should turn deep blue if there is any residual, unconverted starch present in the solution. The solution is brown-red coloured for partially degraded starch, while it is clear for totally degraded starch.
- Measure the absorbance with a spectrophotometer at 620nm.
- Carry out the same procedure for the other starch solutions buffered at different pHs.

3.Effect of Temperature

- Obtain hot water from either a faucet or a hot temperature bath. Adjust the temperatures of the temporary water baths in 500 ml beakers so that they range from 30 ºC to 90 ºC in increments of 10 ºC.
- Prepare the starch substrate by diluting the 20g/l starch solution with an equal volume of pH 7.0 phosphate buffer solution. This results in a working starch concentration of 10 g/l. Add 5 ml of the starch solution to each of the test tubes.
- Allow the temperature of each of the starch solutions to come to equilibrium with that of the water bath.
- Add 0.5 ml of the bacterial amylase solution to each of the thermostated test tubes to start the reaction.
- Stop the reaction after exactly 10 minutes and analyse the starch content by following the procedures.

4.Effect of Heat Treatment

- Place 0.5 ml of the bacterial amylase solution each of eleven test tubes.
- Heat-treat the enzyme solution by placing all the test tubes, except one, in a hot (90ºC) water bath. The untreated enzyme is used as the control. Take out the first test tube from the heat after one minute and quickly bring it to room temperature by immersing it in a cool water bath. Remove the second test tube after 2 minutes, the third after 3 minutes, and so on.
- Add 5 ml of the 10 g/l buffered (pH 7.0) starch solution to each of the test tubes containing the enzymes.
- Carry out the hydrolysis reaction at room temperature and analyse the sample after exactly 10 minutes.
- Mix an equal volume of the $CaCl_2$ solution to the enzymes and repeat the same procedures to investigate the heat stabilization of the enzymes in the presence of Ca^{2+} ions.
- This set of studies can be done quickly if the procedures are synchronized. If time permits, try 0.5 ml samples of the amyloglucosidase and 0.5 ml samples of the fungal amylase solution. Compare the sensitivity to heat for these related enzymes.

5.Activity of Human Salivary Amylase

Obtain enough saliva to repeat the pH effect study.

6.Enzyme Specificity

Use 0.5 ml of the cellulose. Follow a similar procedure to determine the decrease in the starch concentration. Measure the rate with buffered starch solution at pH 4.0 and 7.0.

7.Effect of Substrate Concentration

- Add 0.5 ml of the bacterial amylase solution to 50 ml of a 10g/l starch solution buffered at pH 7.0. The objective here is to slow down the reaction so that multiple sampling is possible with reasonable accuracy before all the starch is consumed.
- Take samples periodically to monitor both the decrease in the starch concentration and the increase in the reducing sugars until most of the starch is hydrolyzed.
- Continuously monitor the viscosity of the substrate-enzyme mixture with a viscometer. Generate a calibration curve for the viscosity as a function of the starch concentration.

8.Effect of Enzyme Sources

- Repeat Procedure 7 with 0.5 ml of the fungal amylase solution.
- Repeat Procedure 7 with 0.5 ml of the amyloglucosidase.
- Repeat Procedure 7 to study the joint action of a mixture of 0.167 ml of bacterial amylase, 0.167 ml of fungal amylase solution, and 0.167 ml of amyloglucosidase.
- This entire Procedure 8 can be concurrently carried out along with Procedure 7.

9.Sequential Enzymatic Treatment (Corn Syrup Production)

In making industrial sugars, for example corn syrup, large gelatinized starch molecules are first chopped into smaller dextrins with the help of bacterial amylase. The liquefaction step is followed by saccharification with either fungal amylase or amyloglucosidase, depending on the end use of the sugar.

- Add 0.5 ml of the bacterial amylase solution to 50 ml of the 20g/l non-buffered starch solution prepared in Step 1. Periodically place a few drops of the reaction mixture on a glass plate and add one drop of the iodine reagent. The colour should finally turn red, indicating the total conversion of starch to dextrin. This liquefaction step should last for approximately 10 minutes.
- When the process of liquefaction is complete, adjust the pH of the starch solution to 4.7 with 1N HCl.

NOTES

- Filter the starch solution if it is turbid. Separate the solution into two equal parts.
- To the first starch solution, add 0.5 ml of amyloglucosidase; to the second solution add 0.5 ml of fungal amylase solution.
- Measure the sugar concentrations periodically. Note that you need to use the appropriate calibration curves because one is maltose and the other is glucose. Also do not forget to reference your observation to the initial absorbance at the start of the saccharification process so that the increase in the sugar concentration can be correctly measured. This saccharification step should last for about 30 to 60 minutes.
- Taste the two sugar solutions and compare the sweetness.

10. Inhibition

Follow Procedure 7, except that the buffered starch solution at pH-7.0 also contains hydrogen peroxide at a level of 0.5 g/l. If time permits, try hydrogen peroxide at a level of 1.0 g/l.

11. Enzyme Activity versus Enzyme Concentration

Mix 0.5, 1.0, 1.5, 2.0, and 2.5 ml of enzyme solutions with 5 ml of 10g/l starch solution. Measure the starch concentration after 10 minutes.

Expected Results

1. **Positive Test:** A clear zone around the line of growth after addition of iodine solution indicates that the organism has hydrolyzed starch.
2. **Negative Test:** A blue, purple, or black coloration of the medium (depending on the concentration of iodine).

Uses

It helps in the differentiation of species of genera *Corynebacterium*, *Clostridium*, *Bacillus*, *Bacteroides*, *Fusobacterium*, and members of *Enterococcus spp*.

Limitations

- It is recommended that biochemical, immunological, molecular or mass spectrometry testing be performed on colonies from pure culture for complete identification.
- Colonies cannot be subcultured from the medium after the addition of Gram's iodine due to the oxidative nature of the reagent and the resulting cell death.

3. HYDROLYSIS OF PROTEIN

Protein hydrolysis is carried out by chemical and enzymatic methods. Most of the enzymes used for protein hydrolysis are from animal sources, such as pancreatin and pepsin, plant sources, such as papain from papaya, ficin from fig, and bromelain from pineapple, and microbial sources, such as Alcalase.

NOTES

Proteolytic enzymes hydrolyze proteins at the optimum temperature and pH and usually target specific peptide cleavage bonds, resulting in digestion consisting of amino acids and peptides of varying size.

Enzymes from animal sources are more specific to their site of action compared to plant enzymes, which are more broadly specific in their action. For example, the enzyme pepsin will cleave at the phenylalanine or leucine bond.

Papain has a broad specificity, cleaving bonds at phenylalanine, arginine, and lysine. Pancreatin cleaves at tryptophan, arginine, tyrosine, leucine, phenylalanine, and lysine bonds.

Proteins incubated with microbes lead to hydrolysis by fermentation, during which proteolytic enzymes are secreted. Proteases from microbial sources offer a wide variety of enzyme activities.

Proteases from bacterial, algal, fungal, and yeast sources are produced on a large scale and usually only require simple purification steps, which can be used for an industrial application such as the production of peptone.

Uses

Protein hydrolysis is a useful route to the isolation of individual amino acids. Examples include cystine from hydrolysis of hair, tryptophan from casein, histidine from red blood cells, and arginine from gelatin.

Common hydrolyzed products used in food are hydrolyzed vegetable protein and yeast extract, which are used as flavour enhancers because the hydrolysis of the protein produces free glutamic acid. Some hydrolyzed beef protein powders are used for specialized diets.

Protein hydrolysis can be used to modify the allergenic properties of infant formula. Reducing the size of cow milk proteins in the formula makes it more suitable for consumption by babies suffering from milk protein intolerance.

Hydrolyzed protein is also used in certain specially formulated hypoallergenic pet foods, notably dog foods for dogs and puppies that suffer from allergies caused by certain protein types in standard commercial dog food brands. The protein contents of the foods are split into peptides which reduces the likelihood for an animal's immune system recognizing an allergic threat. Hydrolyzed protein diets for cats are often recommended for felines with food allergies and certain types of digestive issues.

EXPERIMENT 7: IDENTIFICATION OF PREPARED SLIDES

Mould - *Mucor, Rhizopus, Aspergillus, Penicillium*, Yeast

Bacteria – *Bacilli*

I. Identification of Prepared Slides of Mould - *Mucor, Rhizopus, Aspergillus, Penicillium*, Yeast

The fungi are spore-bearing Eukaryotic organisms without chlorophyll and having absorptive nutrition. These reproduce sexually as well asexually. Primarily, these

are terrestrial microorganisms, though some are present in aquatic environment also, both in marine and fresh water. Many are pathogenic to plants, animals and humans.

The fungi comprise a diverse group of organisms that are heterotrophic and typically saprozoic. In addition to the well-known macroscopic fungi (such as mushrooms and molds), many unicellular yeasts and spores of macroscopic fungi are microscopic. For this reason, fungi are included within the field of microbiology.

Fungi are important to humans in a variety of ways. Both microscopic and macroscopic fungi have medical relevance, with some pathogenic species that can cause mycoses (illnesses caused by fungi). Some pathogenic fungi are opportunistic, meaning that they mainly cause infections when the host's immune defences are compromised and do not normally cause illness in healthy individuals. Fungi are important in other ways. They act as decomposers in the environment, and they are critical for the production of certain foods such as cheeses. Fungi are also major sources of antibiotics, such as penicillin from the fungus *Penicillium*.

Structure of Fungi

The vegetative structure of a fungus is called thallus. It varies in complexity and size, ranging from unicellular yeasts to multicellular moulds. A yeast is a unicellular fungus, producing moist to waxy colony in culture. It is about 5-10 times larger than bacteria and reproduces asexually by budding or transverse division and sexually through spore formation. A mould produces leathery, cottony or profuse powdery growth on medium. It consists of a mass of intercoiling branched, thread like structures called hyphae. The mass is known as mycelium. Depending on the fungal type, the hyphae may be septate, i.e., have cross wall or septum to produce multi cells or aseptate, i.e., septum is absent and the cytoplasm is coenocytic. The hyphae grows on or within the surface of nutrient media to get nutrients. This represents vegetative mycelium. Some of the specialized hyphae arise upward away from the medium surface. It is called the aerial mycelium on which the reproductive structure or spores are formed.

Characteristics of Fungi

Fungi have well-defined characteristics that set them apart from other organisms. Most multicellular fungal bodies, commonly called molds, are made up of filaments called hyphae. Hyphae can form a tangled network called a mycelium and form the thallus (body) of fleshy fungi. Hyphae that have walls between the cells are called septate hyphae; hyphae that lack walls and cell membranes between the cells are called non-septate or coenocytic hyphae.

NOTES

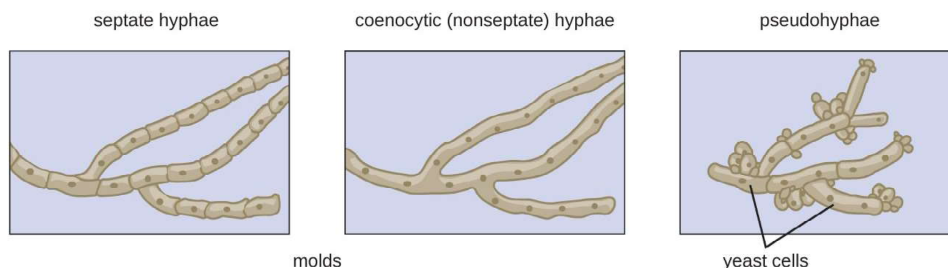


Fig. 1 Multicellular Fungi (Molds) form Hyphae, Which May be Septate or Non-Septate. Unicellular Fungi (Yeasts) Cells form Pseudohyphae from Individual Yeast Cells.

Classification of Fungi

The fungi, is placed in a separate kingdom called Myceteae. Traditionally, fungi were divided into four sub-divisions based on the mode of sexual reproduction as – *Zygomycota*, *Ascomycota*, *Basidiomycota* and *Deuteromycota*. Now, molecular microbiologists have put *Deuteromycotina* among their closed relatives in *Eumycota* (true fungi). The new sub-divisions are *Zygomycota*, *Ascomycota*, *Basidiomycota* and *Chytridiomycota* based on 18s rRNA studies. The different sub-divisions of fungi are; Zygomycota – Fungi belonging to Zygomycota are called Zygomycetes. Hyphae in this sub-class are coenocytic. Asexual spores develop in sporangia at the tips of aerial hyphae. Sexual spores called Zygospores, are formed by fusion of different mating types designated positive and negative, because male and female are morphologically indistinguishable. Zygomycetes are involved in production of foods, like tempeh, sufu. It is also used in commercial preparation of some anaesthetics, birth control agents, industrial alcohol, meat tenderizers, etc. Example: Rhizopus, Mucor.

Deuteromycota – Deuteromycetes or fungi imperfecti reproduce by means of conidia. These have either lost the capacity for sexual reproduction or it has never been observed. Sexual spores if observed in the members of Deuteromycota, it is reclassified into a different genus and placed accordingly into appropriate division.

Fungi imperfecti are important as many of them are human pathogens. Few are important industrially and are involved in antibiotics and food production. Some produce mycotoxins, which are highly toxic and carcinogens to animals and humans. Example: *Aspergillus*, *Penicillium*.

Chytridiomycota are the simplest among true fungi and are commonly called Chytrids. Reproduction is asexual by means of motile zoospores. When sexual reproduction occurs, it results in formation of sporangium. The entire organism is microscopic in size and may consist of a single cell, a small multinucleate mass or a true mycelium.

NOTES

Characteristics of Some Common Fungi

1. *Rhizopus*

Classification

Kingdom *Mycetae*

Division *Amastigomycota*

Class *Zygomycetes*

Order *Mucorales*

Family *Mucoraceae*

Genus *Rhizopus*

Rhizopus is a common laboratory contaminant. It is a spoiling mould and found frequently on the surface of bread, fruits and vegetables. It can grow as weak facultative parasite under certain conditions, causing soft rot of sweet potato and leak of peach, raspberry, strawberry and some other fungal diseases in animals and human. Various species of *Rhizopus* are exploited industrially for production of cortisone (*Rhizopus stolonifer*), alcohol (*Rhizopus oryzae*), lactic acid and fumaric acid (*Rhizopus oryzae*, *Rhizopus stolonifer*).

Identifying Features of *Rhizopus*

- Macroscopically *Rhizopus* appears as a white cottony mass growing rapidly and spread over entire plate during vegetative phase.
- Microscopically, hyphae are aseptate and coenocytic. There are 3 kinds of hyphae: Stolon, Rhizoids and Sporangiophore.
- Rhizopus usually reproduce asexually, but under unfavourable conditions, sexual reproduction (isogamous) occur resulting in formation of zygospores – thick, black, rough structures.

2. *Mucor*

Classification

Kingdom *Mycetae*

Division *Amastigomycota*

Class *Zygomycetes*

Order *Mucorales*

Family *Mucoraceae*

Genus *Mucor*

Mucor is present as a food contaminant. It resembles *Rhizopus* in life history and colonies appear just like *Rhizopus*.

Characteristic Features of *Mucor*

- Stolons characteristics of *Rhizopus* are absent in *Mucor*. The aerial mycelium in *Mucor* consists of branched hyphae, which grow over the surface.

- Rhizoids are absent in *Mucor*.
- In *Mucor*, sporangiophores arise singly instead of a cluster of two or more sporangiophores as in case of Rhizopus.
- Mucor has; Non-septate mycelium, single sporangiophore that arises at a point, globular sporangium containing a columella, oval spores and no rhizoids.

NOTES

3. *Aspergillus*

Classification

Kingdom *Mycetae*

Division *Amastigomycota*

Class *Ascomycetes*

Subclass *Plectomycetidae*

Order *Eurotiales*

Family *Eurotiaceae*

Genus *Eurotium* (*Aspergillus*)

Form Class *Deuteromycetes*

Form Order *Moniliales*

Form Family *Moniliaceae*

Form Genus *Aspergillus*

Aspergillus is a common laboratory contaminant. Its conidia are usually present in air. *Aspergillus* species are used industrially for manufacturing citric, gluconic and gallic acid and for fermenting soy sauce. Wide range of enzymes is produced by *Aspergillus niger* and *Aspergillus oryzae*. *Aspergillus sp*. also causes various diseases of plants.

Different species of *Aspergillus* like, *Aspergillus fumigatus* is responsible for aspergillosis in human. Certain *Aspergillus* species, for example *Aspergillus flavus* produces aflatoxins, which are highly toxic, and carcinogens in human, causing liver cancer. Aspergillus species are also responsible for spoilage of food, leather, cotton fabric, etc., thus reducing their commercial value. It can grow on decaying vegetables, butter, ghee, bread, rice, jam, jellies, etc. easily by producing large number of enzymes.

Identifying Features of *Aspergillus*

- Macroscopically *Aspergillus* colonies are powdery and are of different colours like green, blue, black, yellow, brown, etc.
- Microscopically mycelium consists of branched, bright or pale coloured hyphae some of which grow within the substrate while others grow on the substrate.

NOTES

- From these vegetative hyphae, long, unbranched, non-septate erect hyphae arise called conidiophores. The cell from which conidiophore arise is called foot cell. It is thick walled and T-shaped and one conidiophore arises from each foot cell.
- Conidiophores terminate into a globular structure called vesicle.
- Around the vesicle, there are 1-2 layers of flask shaped structures called phialides or sterigmata.
- Sexual Reproduction occurs by formation of ascus and ascospores.

4. *Penicillium*

Classification

Kingdom *Mycetae*

Division *Amastigomycota*

Class *Ascomycetes*

Subclass *Plectomycetidae*

Order *Eurotiales*

Family *Eurotiaceae*

Genus *Talaromyces* (*Penicillium*)

Form Class *Deuteromycetes*

Form Order *Moniliales*

Form Family *Moniliacea*

Genus *Penicillium*

Penicillium is cosmopolitan in distribution. It is called green or blue mould (look of growth or media above) though exists in different colours. *Penicillium* is used in industries for production of organic acids like oxalic, fumaric and citric acid. It is also a source of antibiotics like Penicillin and Griseofulvin which are produced by *Penicillium chrysogenum* and *Penicillium griseofulvin*.

Identifying Features of *Penicillium*

- Mycelium consists of colourless, septate and branched hyphae, some of which grow inside the substratum to get nutrients and the rest spread on the surface. Former are called haustoria hyphae.
- Erect, tubular septate hyphae called conidiophores grow outward in the air from any cell of the mycelium. No foot cells are present in *Penicillium*. Only one conidiophore arises from one cell.

NOTES

- Unlike *Aspergillus*, conidiophores branches once, twice or even more times to produce primary, secondary or tertiary branches. The ultimate branches bear tufts of flask shaped structures called sterigmata (phialides).
- The conidia are tiny, uninucleate and unicellular, globose, solid, elliptical or pyriform structures. These may be smooth or rough.
- Sexual reproduction is observed in a few species by formation of asci containing ascospores. Ascospores are uninucleate, lens shaped structures.

Objectives

- To identify the various classes of fungi and major features among them
- Identify representatives from 3 categories of fungi:
  - o Basidiomycetes (representative: mushrooms)
  - o Ascomycetes (representative: *Penicillium*, *Saccharomyces*, various dermatophytes)
  - o Zygomycetes (representatives: *Rhizopus*)

Materials Needed

- Culture of *Saccharomyces cerevisiae*
- Prepared slides of *Rhizopus*, *Penicillium*, *Aspergillus*, and *Candida albicans*
- Fresh cultures of fungi on agar plates (*Rhizopus*, *Penicillium*, *Aspergillus*)
- Fresh Agaricus mushrooms

Procedure

Rhizopus Prepared Slides

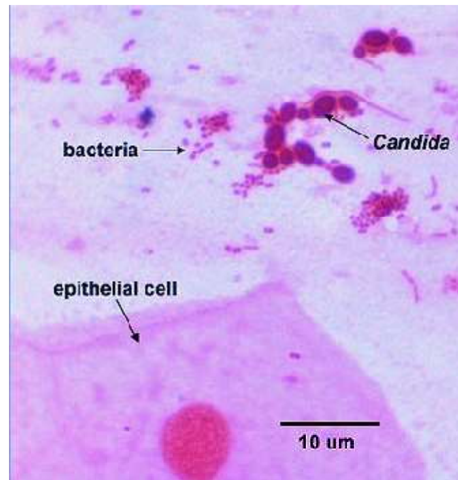
If two different strains (called **'+'** and **'–'** strains) are placed together on a culture medium (or in nature), the hypha will grow towards each other and conjugation will occur. This produces a sexual spore called a zygospore—a diploid sexual spore.

- On 10X and 40X, identify hyphae, sporangia, and sporangiospores.
- Differentiate between the sexual zygospores and the sporangiospores on the slides.

Penicillium* and *Aspergillus

- On 10X and 40X, identify hyphae, conidia fruiting structures, and the asexual conidiospores.

Images Observed Under the Microscope



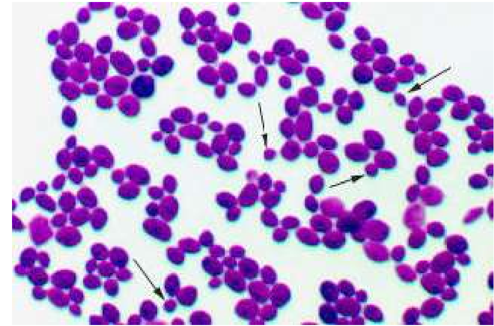
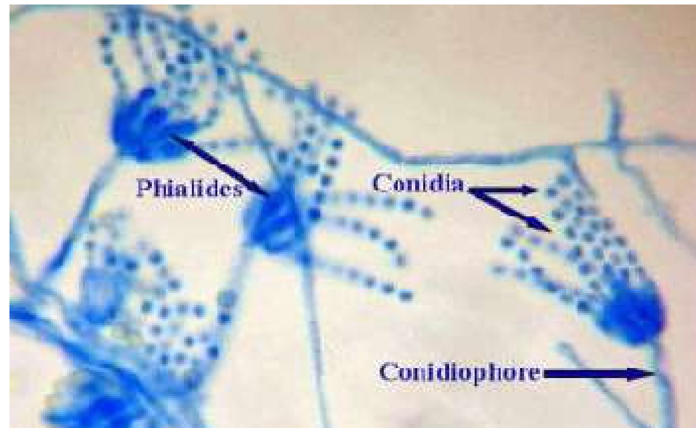


Fig. 2 *Candida albicans Saccharomyces cerevisiae, Showing Budding Cells*



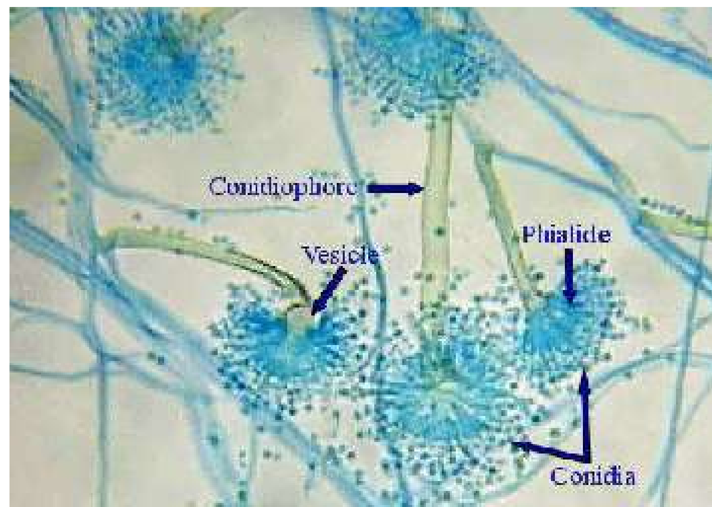


Fig. 3 *Penicillium, Aspergillus*

NOTES



Fig. 4 *Aspergillus*

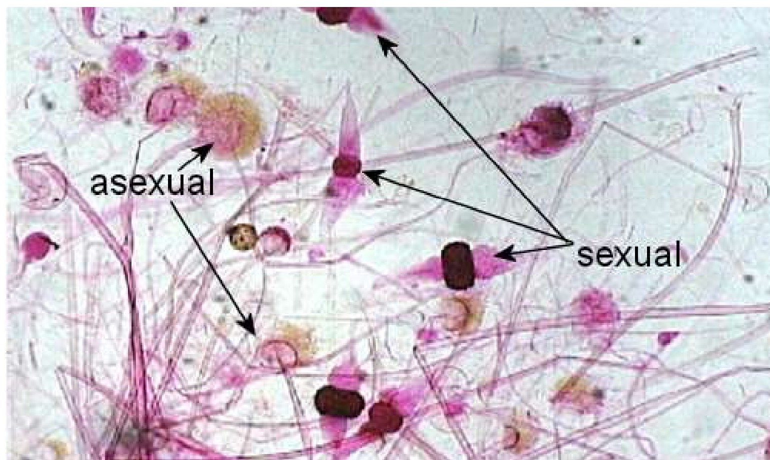


Fig. 5 *Asexual Spores of Rhizopus, Along with Sexual Spores*

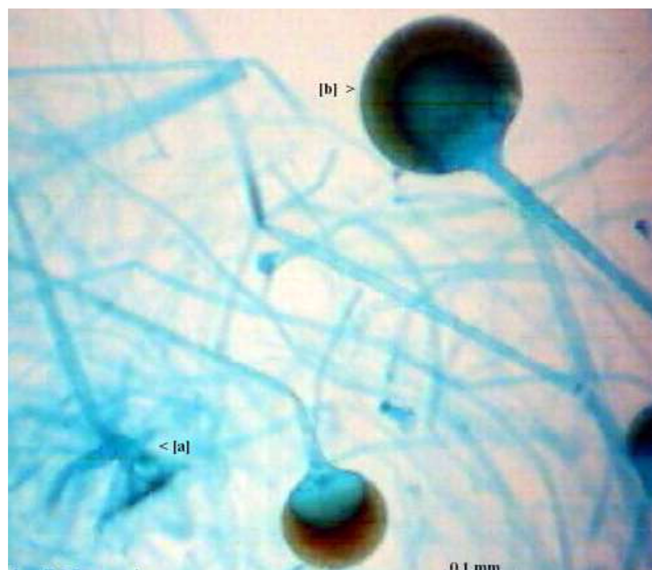


Fig. 6 *Rhizopus sporangia with Asexual Sporangiospores Within*

NOTES

Observation

1. Make labelled diagrams of the structures you observed.
2. Record your observation in the following table:

Fungi	**Observation (Morphological Characteristics)**			**Diagram**
Mucor				
Rhizopus				
Aspergillus				
Penicillium				
Yeast				

II. Identification of Prepared Slides of Bacteria – *Bacilli*

Archean, bacteria are prokaryotic cells are single-celled organisms without a nucleus membrane (nuclear envelope). While they are very small, they are diverse and vary in shape and size.

Bacilli is a taxonomic class of bacteria that includes two orders, Bacillales and Lactobacillales, which contain several well-known pathogens, such as *Bacillus anthracis* (the cause of anthrax). *Bacilli* are almost exclusively Gram-positive bacteria.

The name *Bacillus*, capitalized and italicized, refers to a specific genus of bacteria. The name *Bacilli*, capitalized but not italicized, can also refer to a less specific taxonomic group of bacteria that includes two orders, one of which contains the genus *Bacillus*. When the word is formatted with lowercase and not italicized, ‘Bacillus’, it will most likely be referring to shape and not to the genus at all.

NOTES

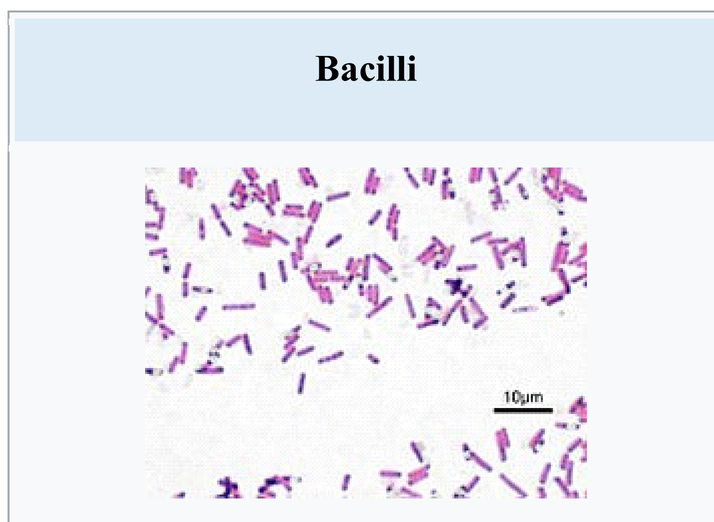


Fig. 7 Bacillus subtilis, Gram Stain

Scientific Classification

Domain: Bacteria

Phylum: Firmicutes

Class: Bacilli

Before preparing for microscopy, bacteria are grown in culture media. This helps enhance cell division and thus increase their numbers. Some of the media used include:

Basal Media, for example nutrient broth and peptone water. This is used for such bacteria as Staphylococcus that do not require special enrichment for growth

Selective Media, for example Lowenstein-Jensen media. This type of media is used to grow specific types while prohibiting others.

Transport Media, for example medium. Transport media are particularly useful in instances where the sample has to be preserved. This also prevents the bacteria from overgrowing before it is transported to the desired destination.

Enriched Media, for example with added blood. Enriched media contain a special component added to the media to favour the growth of given bacteria. For instance, the addition of blood in a given media supports the growth of Streptococci.

Slide Preparation

Materials Required

- Bacteria Sample (Preferably Cultured)
- Distilled Water
- Compound Microscope

NOTES

- Bunsen Burner
- Inoculating Loop or Needles
- Microscope Glass Slide
- Dropper
- Wax Marking Pencil
- Clean Glass Slides

Procedure

Smear Preparation

Bacteria being very small makes them difficult to view under a microscope without first staining. Bacteria needs to be firmly attached to a glass slide before staining. There are two important things to consider when preparing a slide for staining:

- The bacteria should evenly and lightly dispersed. If there are too many bacteria on the slide they will form a big glob and you will not be able to see the morphology of the individual cells. Large blobs of cells also do not stain properly and could yield erroneous results from the improper staining.
- The bacteria need to be firmly attached to the slide so they are not washed off during the staining procedures. All procedures that attach the bacteria to the slide result in some morphological changes. The cells typically shrink in size and will exhibit some changes in shape and extra-cellular matrixes.

Smear from Broth

Broth cultures are usually easier to work with because the cells are already diluted in the broth. Be sure to carefully mix the culture tube to suspend the bacteria in the broth.

- Label your slide. Aseptically transfer a loop-full of organism onto the centre of your slide.
- Use the flat part of the loop to smear the broth drop around the slide. Use a spiralling, circular motion to spread out the drop. Because the broth is full of protein, the smear will usually stay spread out and not bead up on the surface of the slide.
- Set the slide aside to air dry. This will take several minutes at least. Do not rush this step.

Smear from Plate

You can scoop a lot of organisms off with your loop. You may want to use an inoculating needle to transfer your organism to the slide. Be sure to use sterile water to dilute your samples. Regular tap water or the de-ionized water in your rinse bottles are often contaminated with bacteria.

- Label your slide. Aseptically transfer a loopfull of sterile water to the centre of the slide. This serves to both dilute your bacteria and give you something to spread around.

NOTES

- Pick a well-isolated colony.
- Prick it with your sterile needle, or slightly scoop the edge of the colony with your sterile loop.
- Place your needle/loop in the centre of the drop and with a spiralling circular motion spread the bacteria on the slide.
- Set the slide aside to air dry. This will take several minutes at least. Do not rush this step.

Fixation

The fixation procedure is the same regardless of smear source, plate or broth. There are two methods of adhering your bacteria to the slide, heat fixation or methanol fixation. Heat fixing is only used with BSL1 organisms. The organisms we will be working with are BSL2, so you will need to use the methanol fixation technique. Heat fixing the slide can create aerosols and with BSL2 organisms, we need to prevent this as much as possible. Methanol fixation causes fewer changes in cellular morphology and creates no aerosols.

Methanol Fixing (BSL2)

- Be sure your slide is totally dry. Set it on the staining rack over the sink.
- Carefully flood the slide with 95% methanol. Let it sit for two minutes.
- Tilt the slide and pour off the methanol. Touch the edge of the slide to a paper towel to wick off the excess methanol.
- Set the slide aside to air dry before staining.

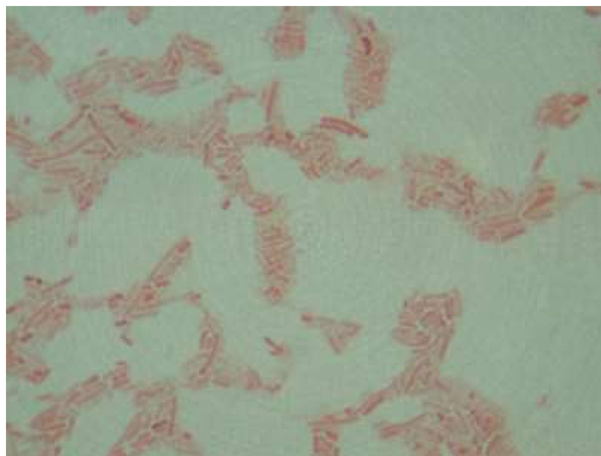


Fig. 8 *Simple Stain*

Simple Stain

For simple stains add one stain to a fixed smear slide, let it sit, rinse it off, let it dry, and view. It is a quick procedure for determining the presence and morphology of bacteria in clinical samples, such as stool and discharges.

Methylene blue is used to determine the morphology of fusiform and spirochetes in oral infections. It is also the stain of choice for identifying the metachromatic granules in *Corynebacterium diphtheriae*. The granules will stain a distinctly deeper blue than the surrounding blue bacteria. Other species of *Corynebacterium* do not have the metachromatic granules. Any basic dyes, such as methylene blue, crystal violet, malachite green, or safranin work well.

Basic (cationic or positively charged) dyes bind to negatively charged components in the cell membrane and cytoplasm.

Materials

- Methylene Blue
- Safranin
- Crystal Violet
- Malachite Green
- Staining Racks
- Micro Tool Boxes
- Prepared Smear Slides

General Considerations

Staining is part art and part science. There are no hard and fast rules for staining and rinsing times.

Simple Stain Procedure

- Place your carefully prepared fixed smear slides on the stain rack over the sink.
  - o Do one slide at a time.
  - o Cover the smear with any of the basic dyes available to you.
  - o You only need enough dye to cover the smear. The stain should not drip off the slide.
- Let the stain sit for 1-5 minutes.
- Using the clothespin, grab the long end of the slide, tilt the slide over the sink and rinse the stain off with a stream of water from the wash bottle.
  - o Be sure to spray above the smear and let it dribble down.
  - o If you spray directly on the smear you are liable to wash the smear off the slide.
  - o Rinse till the water runs clear or is only slightly coloured.
- Touch the edge of the slide to a paper towel to remove excess water. Let it air dry or dry it with blotting paper by placing it in the blotting paper book and pressing lightly. While this method is quicker, you can also blot off a poorly adhered smear.

- View the slide under oil immersion and record your observations.
- Discard the used stained slides in the disinfectant bucket in the sink.

Negative Stain

NOTES

Negative stains are even simpler than simple stains because you do not have to make a smear. A drop of cells is spread on a slide and viewed without fixation. The stain is a suspension of carbon, found in India ink. The carbon particles are negatively-charged, as is the cell membrane. The background looks black or sepia coloured and the cells remain clear, since they repel the dye.

Some positively charged inclusion bodies, such as sulphur, may stain. This stain gives accurate information on cell morphology and capsule presence because the cells are not fixed. Cell size appears slightly larger because any extracellular coatings or secretions on the outside of the cell membrane also do not stain. Negative stains are useful for rapid determination of the presence of *Cryptococcus neoformans*, the causative agent of *cryptococcosis*, in cerebral spinal fluid. This technique is also used when you stain for endospores and capsules.



Fig. 9 *Negative Stain*

Materials Required

- Nigrosin Dye
- Assorted Cultures

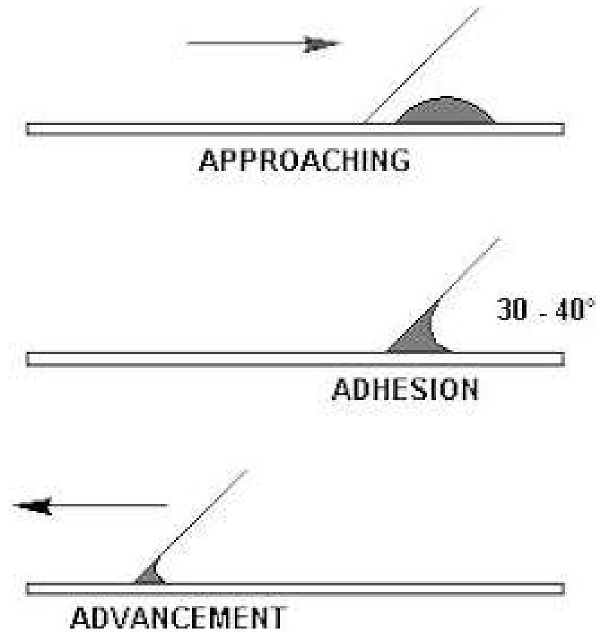
General Considerations

Just as in preparing a smear, you only need a small amount of organism. If you have too many organisms, you won't be able to see the morphology of individual cells. It is also important not use too much ink. If it is too thick, the background will have a cracked appearance similar to mud puddles drying in the sun.

NOTES

Negative Staining Procedure

- Label your slide. If you are working from a broth culture, place a loop-full of organisms about three fourths of the way on the left side of the slide. If you are working from a plate culture, add a drop of sterile water to the slide and dilute your organism in the drop without spreading the drop.
- Put one or two drops of ink on another slide. Use your sterilized loop to pick up a loop-full of ink. Carefully mix it in with the drop of cells, without spreading the drop too much.



- Hold the right end of the slide in your right-hand; with your left–hand take another slide at a 45° or less angle to the first slide, just past your cell drop.
- Scoot the angled slide back along the surface of the first slide till it just touches the drop of ink and cells. Wait for capillary action to draw the liquid along the leading edge of the angled slide.
- Push the angled slide across the surface of the flat slide. Most of the ink should still be left on the original spot. Discard the slide in the disinfectant bucket.
- Set the stained slide aside to air dry before observing it under oil immersion. Be sure to start examining your slide in the area with the faintest grey background.
- Record your observations.
- Discard the stained slide in the disinfectant bucket.

NOTES

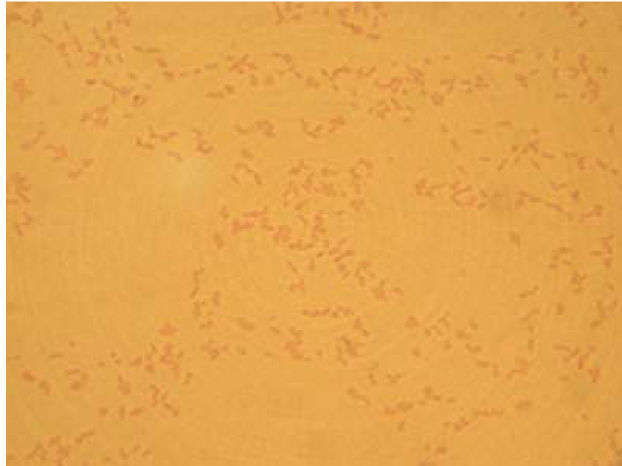


Fig. 10 *Gram Stain (Negative)*

Gram Stain

The Gram stain is the most common differential stain used in microbiology. Differential stains use more than one dye. The unique cellular components of the bacteria will determine how they will react to the different dyes. The Gram stain procedure has been basically unchanged since it was first developed in 1884. Almost all bacteria can be divided into two groups, Gram negative or Gram positive. A few bacteria are gram variable. *Trichomonas*, *Strongyloides*, some fungi, and some protozoa cysts also have a Gram reaction. Very small bacteria or bacteria without a cell wall, such as *Treponema*, *Mycoplasma*, *Chlamydia*, or *Rickettsia* do not have a gram reaction. The characterization of any new bacteria must include their Gram reaction.

Typically a differential stain has four components; the primary stain, a mordant that sets the stain, a decolorizing agent to remove the primary stain, and a counter stain. In the Gram stain, the primary stain is crystal violet which gives the cell an intense purple colour. The mordant, iodine, forms a complex with the crystal violet inside the cell wall. The cell is then washed with either Gram's de-colorizer or 95% ethanol. Gram-positive cells will retain the dye complex and remain purple. The dye rinses out in Gram-negative cells. The counter stain, safranin, is used to colour the cells that lost the primary stain, otherwise they would remain colourless and you will not be able to see them.

The large iodine-crystal violet complex is retained within the cell walls of Gram-positive cells because of the molecular structure of the many layers of peptidoglycan in the cell wall. There are lots of cross-linked teichoic acids and the iodine-dye complex cannot physically get out. There is also less lipid in the membrane and the decolorizing agent cannot get to it as well. Gram-negative cells have an outer membrane and only one layer of peptidoglycan, with more lipid. The crystal violet dye is easily washed out.

NOTES

Gram Stain Procedure

- Label your slide. Prepare your smears on a slide with a Gram-negative on the left, your unknown in the middle, and a Gram-positive on the right.
- Laying your slide on the staining rack, cover the smears with crystal violet for 1 minute.
- Use just enough stain to fully cover the smear but not so much that it runs or drips off the slide.
- Tilt the slide and pour the crystal violet off and briefly rinse with water from your wash bottle.
- Remember not to spray directly on the smears or you will wash them off.
- Flood the slide with iodine mordant for 1 minute.
- Tilt the slide, pour off the excess iodine and gently decolorize with Gram's de-colorizer until it just begins to run clear.
- Tilt your slide on some paper towels to remove excess de-colorizer.
- Flood your slide with the safranin for 1 minute.
- Tilt the slide to pour off the excess safranin and gently rinse with water until it runs clear.
- Let the slide air dry
- Observe under oil immersion.
- The Gram-positive control should be purple and the Gram-negative control should be pink.
- Discard your used slide in the disinfectant bucket.

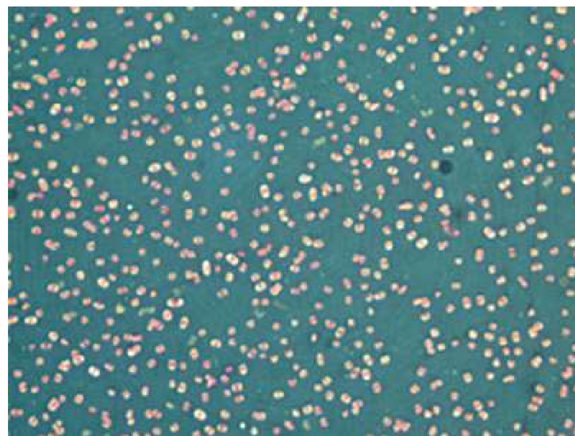


Fig. 11 *Capsule Stain (Klebsiella)*

Result

Depending on the sample under investigation, you will observe and identify the size and shape of the bacteria. They are categorized according to their shape (Morphology) and the how they stain (Gram-positive and Gram-negative bacteria).

Morphology

There are several types based on their general appearance (shape) including:

Cocci (coccus) bacteria are some of the most common bacteria. They are spherical (or ovoid at times) in shape and are divided into; diplococcus (occur in pairs, such as *Neisseria spp.*), streptococcus (occur as a long chain or cells, such as *Streptococcus pneumoniae*) and staphylococcus where they occur in clusters, for example *Staphylococcus saprophyticus*. Cocci may also occur in tetras or in packets of 8 to form a structure that appears like a cube, such as the Sarcina bacteria.

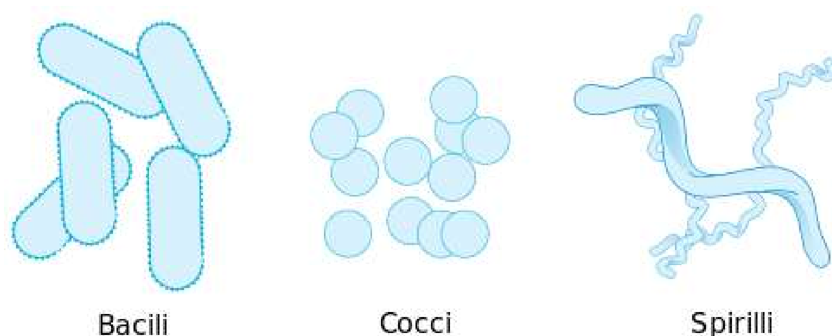


Fig. 12 *Morphological Forms of Bacteria*

Bacillus

Unlike coccus bacteria, bacillus appears as elongated rods (rod-like) when viewed under the microscope. In most cases, the bacilli occur as single cells, for example *Mycobacterium tuberculosis*, but may occur in pairs (diplobacillus) or form chains commonly referred to as streptococcus, for example *Bacillus cereus*).

Spirilla and Vibrio

Vibrio bacteria appear comma shaped whereas Spirilla appear spiral in shape. While some people may confuse the two when viewed under the microscope, they are different when students compare them under high magnification. Example of Vibrios includes *Vibrio vulnificus* and *Vibrio harveyi* while some examples of Spirilla include members of the *Campylobacter* species.

BLOCK III
FOOD BIOTECHNOLOGY

NOTES

EXPERIMENT I: DETERMINATION OF pH OF DIFFERENT FOODS USING pH METER

Aim

To determine pH of different foods using pH meter.

Principle

Determination of pH of foods is important for production of safe and high quality food, since it is directly correlated to the physical and chemical reactions occurring during food processing. For example, gel formation in preparation of jams and jellies; fermentation process in production of cheese and so on. Food spoilage results in change in pH in foods and thus reduces their shelf life.

pH is the measure of the concentration of hydrogen ions (i.e., acidity) in a solution. A pH electrode is used to measure the pH of the food in liquid form or its homogenate. In case of solid foods pH can be measured at the surface of the food sample. In either case, pH value is obtained which is expressed as a number on a scale from 0 to 14.

Materials Required

- Equipment: pH Meter with pH Electrode (General Purpose and Surface Probe)
- Thermometer (Digital or Alcohol)

Reagents

- pH Standard Buffers [4.0 +/- 0.01 (Temperature 20°C); 7.0 +/- 0.01 (Temperature 20°C); 10.0 +/- 0.01 (Temperature 20°C)]
- Phosphate Buffered Solution (PBS)
- Distilled Water (DW)
- Storage Solution
- Filling Solution
- Cleaning Solutions

Methodology

Calibration of pH Meter

- Place the pH 7 and pH 4 or pH 10 reference buffers in a plastic bottles.
- Rinse a digital or alcohol thermometer with a stream of deionised water and use a tissue to remove excess water.
- Immerse the thermometer in the pH 7 buffer, stir gently and wait 1 or 2 minutes for thermal equilibrium. Read the temperature and remove.

NOTES

- Adjust the temperature on the secondary LCD of the pH meter with the arrow keys '∆ºC' or '∇ºC' to the temperature displayed on the thermometer.
- Rinse the pH electrode with a stream of deionised water and use a tissue to remove excess water.
- Immerse the electrode into the pH 7 buffer, stir gently and wait 1 or 2 minutes for thermal equilibrium.
- Press CAL. The 'CAL' and 'BUF1' indicators and the most common '7.01' buffer will be displayed on the secondary LCD.
- If necessary, press '∆ºC' or '∇ºC' to select a different buffer value.
- The 'NOT READY' indicator will blink on the LCD until the reading has stabilised.
- When the reading is stable and close to the selected buffer, 'READY' and 'CFM' will blink.
- Press CFM to confirm calibration.
- The meter stores the reading and the calibrated value is then displayed on the primary LCD and the secondary LCD will display the second expected buffer value.
- Rinse the pH electrode with a stream of deionised water and use a tissue to remove excess water.
- Immerse the electrode into the 2^{nd} buffer (use pH 4 if measuring in the acid range or pH 10 if measuring in the alkali range) and stir gently.
- If necessary, press '∆ºC' or '∇ºC' to select a different buffer value.
- The 'NOT READY' (pH 211) or (HI2122) indicators will blink on the LCD until the reading has stabilised.
- When the reading is stable and close to the selected buffer, 'READY' and 'CFM' will blink.
- Press CFM to confirm calibration. The meter stores the reading and returns to normal operational mode, completing the calibration.

Determination of pH in Food Samples

Sample Preparation Liquid Foods

Using aseptic technique remove a portion of the sample to a sterile honey jar/ universal, avoiding contamination of the bulk of the food.

Sample Preparation Solid Foods

- Surface probe: No sample preparation required. Select an area of food that is regular if not consider cutting it to expose a flat surface
- Liquid probe: 1:10 homogenate of the food is prepared.
- If the pH is not measured immediately, store the sample in a refrigerator and test within 24 hours of homogenisation.

NOTES

Measuring pH

- Make sure the appropriate standard buffers have been tested and the instrument(s) has been calibrated before performing pH measurements.
- Press the ON/OFF key.
- Remove the probe from the storage solution and rinse the electrode thoroughly with deionised water to remove salt deposits.
- Blot dry with clean tissue.
- Place the probe into the food sample or apply to the solid surface of the food ensuring full contact with the flat probe end.

Observation

Wait for the reading to settle before recording the result.

The machine automatically displays the result.

Precautions

- Rinse the probe with deionised water before continuing with the next sample.
- The pH electrode should be returned to the storage solution after use and the pH meter should be switched off at the end of each day.
- In use solution should not be used for more than 2 weeks.
- Write the date of expiry on the bottles.
- Unused pH solution must never be transferred back into the stock bottle.

Reference pH Values

Low Acid (pH 7.0 -5.5)

- Milk 6.3-6.5
- Cheddar cheese 5.9
- Roquefort cheese 5.5-5.9
- Bacon 5.6-6.6
- Red meat 5.4-6.2
- Ham 5.9-6.1
- Canned vegetables 5.4-6.4
- Poultry 5.6-6.4
- Fish 6.6-6.8
- Crustaceans 6.8-7.0
- Butter 6.1-6.4
- Potatoes 5.6-6.2
- Rice 6.0-6.7
- Bread 5.3-5.8

NOTES

Medium Acid (pH 5.5-4.5)

- Fermented vegetables 3.9-5.1
- Cottage cheese 4.5
- Bananas 4.5-5.2
- Green beans 4.5-5.5

Acidic (pH 4.5-3.7)

- Mayonnaise 3.0-4.1
- Tomatoes 4.0

High Acid (pH <3.7)

- Canned pickles & fruit juices 3.5-3.9
- Sauerkraut 3.1-3.3
- Citrus fruits 3.0-3.5

EXPERIMENT II: STUDY QUALITY CHARACTERISTICS OF FOODS PRESERVED BY DRYING/DEHYDRATION/ FREEZING

Aim

To Study quality characteristics of foods preserved by drying/dehydration/ freezing.

Principle

Drying of food varieties is an old practice that has been embraced to safeguard food varieties past their regular time span of usability. This evolved from exposure of food varieties to the sun, to separate from them an excessive amount of the water, along these lines contributing for their preservation. Apart from these, hot air drying, spray drying, lyophilisation (freeze drying), infrared, microwave or radiofrequency drying, osmotic pressure drying are many more advanced methods of drying. Many food varieties can be safeguarded through drying, yet their organoleptic properties are particularly adjusted when contrasted with their fresh forms. Drying includes the utilization of heat to disintegrate the water present in the food, and furthermore the expulsion of the steam from the food surface. Consequently, it consolidates heat and mass exchange for which energy should be provided.

In sun based drying the source of energy is the Sun. In this way it is an extremely modest drying technique, however then again it has numerous disadvantages in light of the fact that the food is presented to pollution sources (insect infestation, birds and different creatures) and is additionally emphatically susceptible to climate conditions. The convective drying of permeable media, including food sources, has an essential part in a few mechanical applications. Attributable to its high accessibility and efficiency, it is one of the most popular methods. Spray drying is a generally utilized procedure to change over a fluid state into a powder structure. The fluids go through an atomizer or spray and are converted

NOTES

into smaller particle size (10-200 μm). The utilization of radiofrequency energy for dielectric warming of food materials is a significant application region, which has been concentrated as a potential technique for drying agrarian items. The radiofrequency heating tends to be direct in nature, so its inside is warmed quicker than its surface. The water is released without overheating or parchedness of the surface. Osmotic drying depends on the rule that when cell materials are inundated in a hypertonic medium, a main thrust for water expulsion sets up due to the higher osmotic pressure resulting in removal of water from organic products or vegetables which are soaked in a sugar or salt solution (which has an osmotic pressure higher than the food).

In lyophilisation/ freeze-drying, the water is first frozen and afterward sublimated, under regulated of pressure factor and temperature. Lyophilization is costly as it involves freezing, the creation of vacuum and the expensive equipment for operation. Lyophilization is utilized in industry for the drying out of food sources like mushrooms, spices, juices, meats, fish etc.

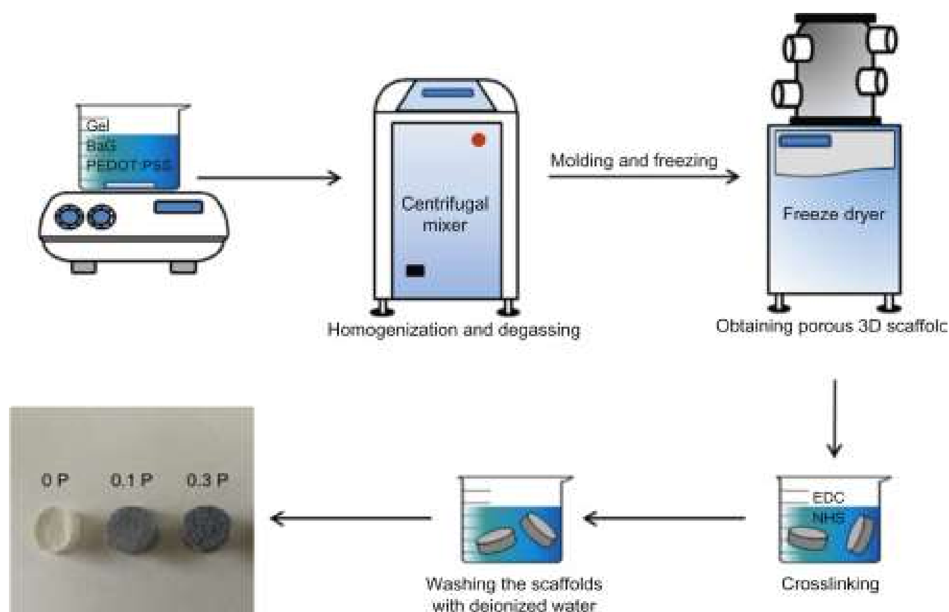


Fig. 1 *Freeze Drying*

Materials Required

- Electric Balance
- Crucibles
- Oven
- Desiccators
- Moisture Boxes

Method

- Take the sample box and weigh it with lid over it.

- Put the sample in it (approximately 5-10 grams).
- Keep the sample in an oven at 105 degree C for 24 hours.
- Take out the sample after 24 hours.
- Cool it in a desiccator for 5 hours.
- Weigh it along with the lid over it.
- Note down the final weight.
- Calculate the difference in weight of the sample.

NOTES

Observation

Fruits and Vegetables	Initial Weight	Final Weight	Overall Appearance	Colour	Aroma	Taste
Apple						
Banana						
Beets						
Carrot Cassava						
Celery Eggplant						
Ginger						
Peppers						
Potatoes						
Mangoes						
Papaya						
Pineapple						
Spinach						
Tomatoes						
Watermelon						
Yams						

Result

1. Calculate the moisture percent in each case:

   $MC = w-d/w * 100$

   w= wet weight

   d= weight after drying

2. Record the overall acceptability and compare within the various fruits and vegetables as given in the above table (you can change the samples as per the seasonal availability of fruits and vegetables).

EXPERIMENT III: TO PERFORM PASTEURIZATION OF FLUIDS USING DIFFERENT METHODS

Aim

To perform pasteurization of fluids using different methods**.**

Principle

Pasteurization is a procedure of uninterrupted heating the milk to an optimal temperature for an optimal time of treatment and cooling to 5°C rapidly. Pasteurization involves deactivation of enzymes and pathogenic organisms present in milk based on the extent of time and temperature treatment. Rapid cooling is significant as it halts the growth of microorganisms.

NOTES

Pasteurization of milk can be carried out by batch method or High Temperature Short Time (HTST). Batch processing involves heating the milk to 62.8°C with the help of steam/hot water for 30 minutes followed by cooling to 5°C in chilled water. High Temperature Short Time (HTST) method is used to heat the milk at 71.7°C (161°F) for 15 seconds in plate heat exchanger.

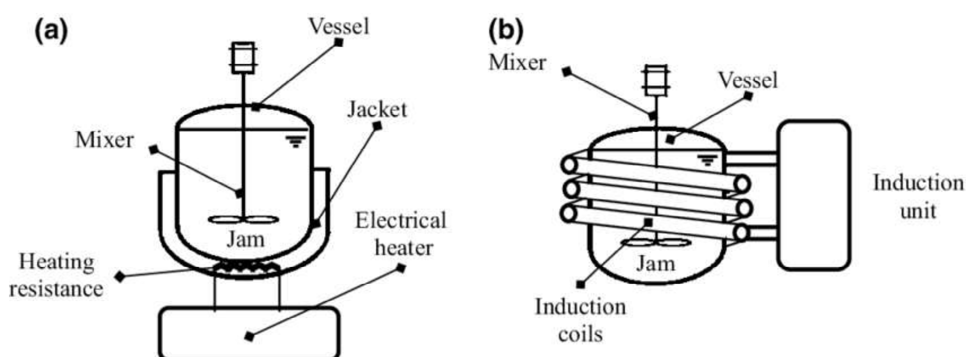
Requirements

- Batch pasteurizer and HTST pasteurizer fitted with all the attachments for holding
- Heating and cooling milk thermometers
- Cans, bottles/pouch filling system
- Detergents
- Sterilizing agents

Methodology

Batch Pasteurization

- Take the desired quantity of milk in a stainless steel holding tank.
- Open the steam valve and heat the milk in the holder or multipurpose tank (vat), while stirring the milk by means of a mechanical agitator
- As soon as the temperature of 62.8°C (145°F) is reached maintain it for 30 minutes.
- Close the steam valve.
- Cool the milk first with cold water and then with chilled water to 4-5°C or below.
- Fill in the cans /bottles /polyethylene pouches.
- Store the pasteurized milk below 4-5°C for delivery to consumers.



NOTES



***Fig. 1** Batch Pasteurization*

HTST Pasteurization

- Clean the plant by circulating detergents, followed by warm water.
- Sterilize the HTST unit by circulating hot water.
- Start the vacuum pump and adjust the vacuum by adjusting the temperature of pasteurizer.
- Circulate hot water and maintain the temperature at 71.7°C (161°F) by regulating the steam valve.
- Pump milk to the balance tank through the clarifier and maintain the level of milk.
- Pump the milk to the regeneration section.
- Set the setting needle, temperature setting needle of the thermograph at a slightly higher temperature, say 73°C (162.5° F), to ensure proper pasteurization.
- Collect the pasteurized milk in a sterilized storage tank or pack in containers.

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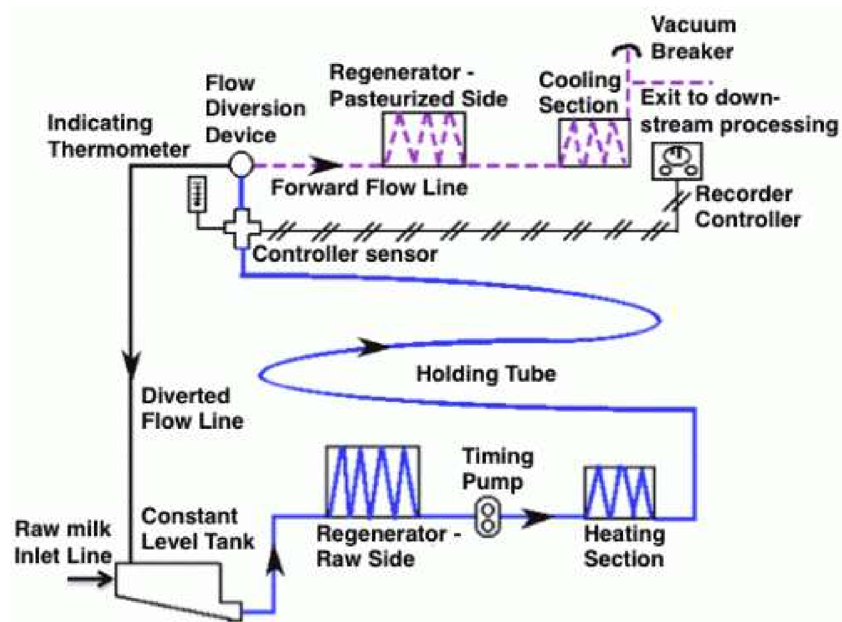




Fig. 2 *HTST Pasteurization*

Observations

Batch Pasteurization

Record the following observations:

- Equipment: details/ cleaning solution/ sterilization details.
- Time taken for sterilization.
- Capacity of the pasteurizer.
- Capacity of the pumps, storage tank.

NOTES

- Temperature of raw milk.
- Temperature of milk after final heating.
- Temperature of milk after cooling.
- Total quantity of milk pasteurized.
- Fat percent of milk.
- Acidity of milk.
- Starting time of heating the milk.
- Holding time of milk at 145 degree F (62.8 degree C).
- Result of phosphate test: positive or negative?
- Result.

HTST Pasteurization

Record the following observations:

- Equipment: details/ cleaning solution/ Sterilization details.
- Time taken for sterilization.
- Temperature of raw milk.
- Temperature of milk after regeneration.
- Temperature of milk after final heating.
- Temperature of milk after cooling.
- Total quantity of milk pasteurized.
- Phosphate test.
- Result.

Precautions

1. Clean, and sterilize the equipment deployed for this experiment using boiling water.
2. Continuous heating of the milk to the specified temperature for the required time should be followed by rapid cooling

EXPERIMENT IV: TO PERFORM BLANCHING OF DIFFERENT PLANT FOODS

Aim

To blanch any given fruits or vegetables; and also determine their blanching time.

Principle

Blanching is a cooking process in which vegetable or fruit, is immersed in boiling water, removed after a time interval, and finally plunged into iced water or under cold running water in order to terminate the cooking process.

NOTES

Blanching is inhibits the enzyme action which can cause loss of flavour, colour and texture.





Fig.1 *Blanching*

Inadequate blanching has a negative effect on quality of finished product Under-blanching fuels up the activity of enzymes and is poorer than no blanching whereas over-blanching causes loss of flavour, colour, vitamins and minerals.

Suitability of blanching is determined by inactivation of enzymes *viz.* catalase and peroxidase; that are present in most of the vegetables and some fruits. Their destruction will consequently lead to inactivation of other significant enzymes.





NOTES

Fig. 2 *Blanching Machine*

Materials Required

- Heating Vessels
- Stainless Steel Sieve
- Weighing Balance
- Measuring Plastic Cylinders
- Heating Plate
- Fruits and Vegetables (Carrots, Beans, Cabbage, Potatoes)
- Distilled Water
- Labels, Markers, Trays
- 1% Guaiacol (in Alcohol) Solution
- Peroxide Solution 0.3% (in Water)

Preparation of Reagents

1% Guaiacol (in alcohol) solution

- 1 g guaiacol is dissolved in about 50 ml of 50% ethyl alcohol
- The volume is then made up to 100 ml with 50% ethyl alcohol.

Peroxide solution 0.3%

- 5ml perhydrol is dissolved in150 ml with distilled water.

NOTES

Procedure

- Wash, drain and sort vegetables and fruits.
- Peel and cut the vegetables into small pieces. Weigh 100g of the prepared vegetable.
- Take 400ml water in a beaker and boil. The ratio of water : vegetables is 4:1.
- Dip the prepared 100g vegetables in to boiling water and start the timer.
- After every 30seconds remove 1g (approx) of vegetables from the beaker and immediately put in cold water.
- Take this 1g of sample, homogenized in a motar-pestle.
- Filter through a muslin cloth and do the tests for the presence of catalase and peroxidase enzyme.
- Drain vegetables thoroughly.
- Pack the vegetables either by dry-pack or tray-pack.
- Dry-pack: Pack vegetable tightly into containers or freezer bags. Press out air and seal tightly.
- Freeze.
- Frozen vegetables will maintain high quality for 8 to 12 months at zero degrees F or lower.

Catalase Presence Test

- Take 1ml of extract and add 1ml of H_2O_2 solution. In the presence of Catalase, a strong oxygen generation (effervescence) is observed for 2-3 minutes.
- $2H_2O_2$ make arrow here 2 $H_2O + O_2$

Peroxidase Presence Test

- To the same tube to which H_2O_2 is added, add 0.5ml guaiacol solution. Keep the tube aside for some time for colour development.
- Appearance of red colour confirms the presence of Peroxidase.
- Continue till red colour with guaiacol ceases to appear.
- The time taken from zero minute to the time when red colour is no more observed is called Blanching time for the vegetable under study.

NOTES

Observation Table

Fruit / Vegetable	Time	Temperature	Catalase Test	Peroxidase Test	Change In Sensory Characteristics of Fruits and Vegetables

Results

Fruit/Vegetable	Optimal Time	Optimal Temperature

Precautions

- Select only microbiologically safe vegetables.
- Remove all spoiled, rotten vegetables/fruits.
- Maintain clean, hygienic working atmosphere

EXPERIMENT V: METHODS OF FOOD SAMPLING AND CONCEPT OF SHELF LIFE OF DIFFERENT FOODS

Aim

To understand methods of food sampling and concept of shelf life of different foods.

Principle

According to FSSAI, 'Sampling involves the selection of a certain portion, number of container and product units from a particular lot of the same food. It must be as representative as possible of the whole consignment or from lot'. Shelf-life is defined as 'the time during which the food product will: remain safe; retain desired sensory, chemical, physical and microbiological characteristics; comply with any label declaration of nutritional data, when stored under the recommended conditions. It begins from the time the food is finished processing and packaged'.

NOTES





Fig. 1 *Food Sampling*

Factors Affecting Shelf Life

- Subsequent heat treatment.
- Use of preservatives in product formulation.
- Composition of atmosphere within packaging.
- Environmental microbial counts.
- Nutrients.
- Available Oxygen Exposure to Light (UV and IR).
- Relative Humidity (RH).
- Redox Potential (Eh).
- pH

- Pressure
- Time–temperature treatment.
- Water activity.

NOTES

Sample Collection

- Clearly define the population that is to be sampled.
- The population may vary in size and extrapolating information obtained from a sample of a production should be done accurately
- Conclusions should be drawn from appropriately on the basis of following 3 parameters:
  - o Collection of representative sample.
  - o Sample preparation.
  - o Analytical methods using suitable methods and devices.

Sampling for Microbiological Examination of Foods

- Sampling plans.
- Level of contamination considered to be o Acceptable (cfu/g < cfu/g).
- Test method (for example: Most Probable number, Standard Plate Count)
- The number of samples to be tested (n) chosen independently or randomly.
- Microbe or groups of microbes of interest.
- Sampling plans.

Method

- Accelerated shelf-life testing: The food product is conditioned and stored at elevated temperature and/or humidity and the quality changes of the product are evaluated at a specific sampling rate.
- It can be two to four times faster than the real shelf life study.
- Real time shelf life testing: Food products are stored under stated or selected conditions for longer than the expected shelf life and check at regular intervals to see when spoilage begins.

Observation

Parameters for measuring shelf life sensory changes

- Appearance, consistency, colour, aroma, taste, texture were analysed by panellists.
- These are usually quantitative quality measures from trained panels.

Sensory Testing Analytical Test

- Hedonic Test
- Difference Test

NOTES

- Quantitative Test
- Simple Descriptive
- Duo-Trio
- Triangle
- Paired Comparison
- Profiling
- Time
- Intensity
- Preference
- Acceptability

Physico-Chemical Analysis

- pH
- Moisture
- Acidity
- Salt content
- TSS

Microbiological Analysis

- Microbial count.
- *E. coli*, Coliform, TPC, Yeast and mold and other bacteria are determined.

Result

Food Item	Parameter to be Tested	Result
1.	Parameters for Measuring Shelf Life Sensory Changes	
	Sensory Testing Analytical Test	
	Physico-Chemical Analysis	
	Microbiological Analysis	
2.	Parameters For Measuring Shelf Life Sensory Changes –	
	Sensory Testing Analytical Test	
	Physico-Chemical Analysis	
	Microbiological Analysis	

Note:

- Shelf life of food vary with different food items, packaging, storage, distribution, etc.
- Accelerated Shelf Life Test (ASLT)
- Real time are two methods for calculation.

Sensory, physico-chemical, microbial analysis used to determine shelf life.

NOTES

Precautions During Sampling

- Inappropriately collected samples results in inappropriate laboratory results.
- Samples must be brought to the laboratory in the original air-tight containers or representative samples in sterile containers under aseptic conditions.
- Clean, dry, leak-proof, wide-mouthed and sterile (hermetically sealed) containers of a size suitable for sample collection should be used.
- Plastic jars or metal cans should be used as compared to glass containers, which may break and contaminate the sample.
- Label all the containers with name of the sample, date of collection, name of sample collector, expiry date, and important instructions.
- Carefully label as the ink might penetrate the container.
- Whenever possible, obtain at least 100 g for each sample.

Quantity of Food Samples to be Collected for Analysis

- Milk 500 ml
- Sterilized Milk 250 ml
- Malai/Dahi 200 g
- Yoghurt/Sweetened Dahi 300 g
- Paneer/Khoya/Shrikhand 250 g
- Cheese/Cheese Spread 200 g
- Evaporated Milk/Condensed Milk 200 g
- Ice-Cream/Softy/Kulfi/Ice Candy/Ice Lolly 300 g
- Milk Powder/Skimmed Milk Powder 250 g
- Infant Food/Weaning Food 500 g
- Malt Food/Malted Milk Food 300 g
- Butter/Butter Oil/Ghee/Margarine/Cream/ Bakery Shortening 200 g
- Vanaspati, Edible Oils/Fats 250 g
- Carbonated Water 600 ml
- Baking Powder 100 g
- Arrow Root/Sago 250 g
- Corn Flakes/Macaroni Products/ Corn Flour/ Custard Powder 200 g
- Spices, Condiments and Mixed Masala (Whole) 200 g
- Spices, Condiments and Mixed Masala (Powder) 250 g
- Nutmeg/Mace 150 g
- Asafoetida 100 g
- Compounded Asafoetida 150 g

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- Saffron 20 g
- Gur/Jaggery, Icing Sugar, Honey, Synthetic Syrup 250 g
- Cane Sugar/Cube Sugar/Refined Sugar/Dextrose, Misri/Dried Glucose Syrup., 200 g
- Artificial Sweetener 100 g
- Fruit Juice/Fruit Drink/Fruit Squash 400 ml
- Tomato Sauce/Ketch Up/Tomato Paste, Jam/ Jelly/ Marmalade/Tomato Puree/Vegetable Sauce 300 g
- Non Fruit Jellies 200 g
- Pickles and Chutneys 250 g

BLOCK IV
BIOSTATISTICS

NOTES

EXPERIMENT I: TABULATION AND GRAPHICAL REPRESENTATION OF DATA

Aim

To tabulate the classified data.

Tabulation of Data

The process of placing classified data into tabular form is known as tabulation. A table is a symmetric arrangement of statistical data in rows and columns. Rows are horizontal arrangements whereas columns are vertical arrangements. It may be simple, double or complex depending upon the type of classification.

Types of Tabulation

Following are the types of tabulation:

- Simple Tabulation or One-Way Tabulation
- Double Tabulation or Two-Way Tabulation
- Complex Tabulation

Simple Tabulation or One-Way Tabulation

When the data are tabulated to one characteristic, it is said to be a simple tabulation or one-way tabulation. For example: Tabulation of data on the population of the world classified by one characteristic like religion is an example of a simple tabulation.

The number of adults in different occupations in a locality

Occupations	No. Of Adults
Total	

Years to repay loan	Monthly repayment (per $1000)
1	$91.25
2	$49.58
3	$35.69
4	$28.75
5	$24.58

Double Tabulation or Two-Way Tabulation

When the data are tabulated according to two characteristics at a time, it is said to be a double tabulation or two-way tabulation. For example: Tabulation of data on

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the population of the world classified by two characteristics like religion and sex is an example of a double tabulation.

Occupation	No. of Adults		Total
	Male	Female	
Total			

	Sport	Gym	Running	Total
Adults	0	2	24	26
Children	3	3	0	6
Total	3	5	24	32

What fraction were Adults that preferred Running?

Complex Tabulation

When the data are tabulated according to many characteristics, it is said to be a complex tabulation. For example: Tabulation of data on the population of the world classified by three or more characteristics like religion, sex and literacy, etc., is an example of a complex tabulation.

Objectives of Tabulation

Following are the objectives of tabulation:

Systematic Presentation of Data: Generally the collected data is in fragmented form. The mass of data is presented in a concise and simple manner by means of statistical tables. Thus, tabulation helps in presenting the data in an orderly manner. It reduces the bulk of information, i.e., it reduces raw data in a simplified and meaningful form so that it can be easily interpreted by a common man in less time.

Facilitates Comparison of Data: If the data is in the raw form, it is very difficult to compare. Comparison is possible when the related items of data are presented in simple and concise form. The presentation of complete and unorganized data in the form of tables facilitates the comparison of the various aspects of the data. The representation of data in rows and columns is helpful in simultaneous detailed comparison on the basis of several parameters.

To Bring Out Essential Features of Data: It brings out the main characteristics of data. It presents facts clearly and precisely without textual explanation.

To Save Space: A table presents facts in a better way than the textual form. It saves space without sacrificing the quality and quantity of data.

Identification of the Desired Values: In tabulation, data is presented in an orderly manner by arranging it in rows and columns. Therefore, the desired values can be identified without much difficulty. In the absence of tabulated data, it would be rather difficult to locate the required values.

Provides a Basis for Analysis: Presentation of data in tabular form provides a basis for analysis of such data. The statistical methodology suggests that analysis follows presentation of data. A systematic presentation of data in tabular form is a prerequisite for the analysis of data statistical tables are useful aids in analysis. Tables serve as the best source of organised data for statistical analysis. The task of computing average, dispersion, correlation, etc., becomes easier if data is presented in the form of a table.

Exhibits Trend of Data: By presenting data in a condensed form at one place, tabular presentation exhibits the trend of data. By looking at a statistical table, we can identify the overall pattern of the data.

Assignment

1. Tabulate the following data using appropriate type of table:
   a. The population of the various states (X,Y, Z) of India in 2011 and 2021 are X (5,06,38,918 and 7,02,38,018), Y(6,93,04,804 and 8,13,04,104) and Z (4,78,17,416 and 5,00,19,226). What inference can you draw from the given data? Tabulate the data for increase in population in various states in the given time period.
   b. Tabulate the marks distribution of students of class X to perform result analysis:

Marks (%)	No. of Students
=<30	45
31 -40	160
41-50	256
51-60	387
61-70	356
71-80	250
81-90	200
91-100	150
Total	1804

   c. The table below shows the education status in the village Pochanpur. Tabulate in terms of :
      i. The percentage composition of male and female in the village
      ii. Employment status (%)

Gender with Employment Status	Composition
Male Employed	1000
Male Unemployed	200
Female Employed	600
Female Unemployed	350
Total	

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GRAPHICAL REPRESENTATION OF DATA

Graphical Representation is a way of analysing numerical data. It exhibits the relation between data, ideas, information and concepts in a diagram. It is easy to understand and it is one of the most important learning strategies. It always depends on the type of information in a particular domain.

General Rules for Drawing Graphs, Diagrams and Maps

- Selection of a Suitable Method
- Selection of Suitable Scale
- Design

Selection of a Suitable Method

Data represent various themes, such as temperature, rainfall, growth and distribution of the population, production, distribution and trade of different commodities, etc. These characteristics of the data need to be suitably represented by an appropriate graphical method. For example, data related to the temperature or growth of population between different periods in time and for different countries/states may best be represented using line graphs. Similarly, bar diagrams are suited best for showing rainfall or the production of commodities. The population distribution, both human and livestock, or the distribution of the crop producing areas may suitably be represented on dot maps and the population density using choropleth maps.

Selection of Suitable Scale

The scale is used as measure of the data for representation over diagrams and maps. Hence, the selection of suitable scale for the given data sets should be carefully made and must take into consideration entire data that is to be represented. The scale should neither be too large nor too small.

Design

We know that the design is an important cartographic task. The following components of the cartographic designs are important. Hence, these should be carefully shown on the final diagram/map.

- **Title:** The title of the diagram/map indicates the name of the area, reference year of the data used and the caption of the diagram. These components are represented using letters and numbers of different font sizes and thickness. Besides, their placing also matters. Normally, title, subtitle and the corresponding year are shown in the center at the top of the map/diagram.
- **Legend:** A legend or index is an important component of any diagram/ map. It explains the colours, shades, symbols and signs used in the map and diagram. It should also be carefully drawn and must correspond to the contents of the map/diagram. Besides, it also needs to be properly positioned. Normally, a legend is shown either at the lower left or lower right side of the map sheet.

- **Direction:** The maps, being a representation of the part of the Earth's surface, need be oriented to the directions. Hence, the direction symbol, i.e., North, should also be drawn and properly placed on the final map

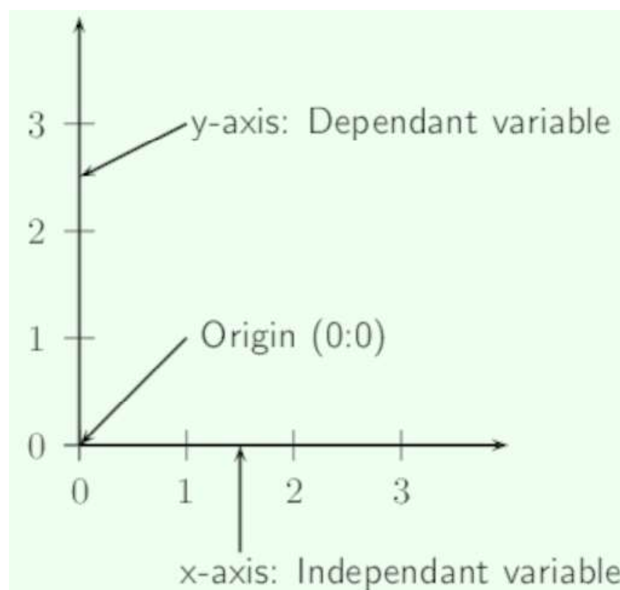
Types of Graphical Representation

There are different types of graphical representation. Some of them are as follows:

Line Graphs

Line graph or the linear graph is used to display the continuous data and it is useful for predicting future events over time. Line graphs are the best type of graph to use when you are displaying a change in something over a continuous range. For example, you could use a line graph to display a change in temperature over time. Time is a continuous variable because it can have any value between two given measurements. It is measured along a continuum. Between 1 minute and 2 minutes are an infinite number of values, such as 1.1 minute or 1.93456 minutes.

Line graphs are used when: The relationship between the dependent and independent variables is continuous. Both dependent and independent variables are measured in numbers.



Features of Line Graph

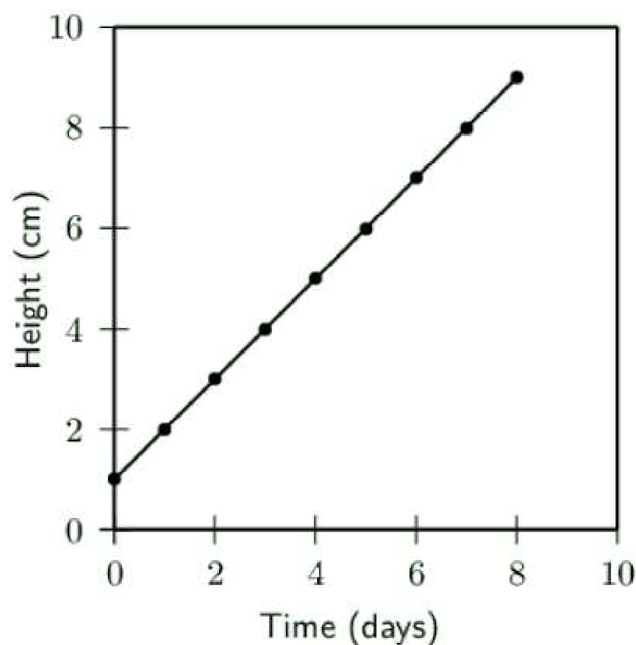
Following are the features of line graph:

- An appropriate scale is used for each axis so that the plotted points use most of the axis/space (work out the range of the data and the highest and lowest points).
- The scale must remain the same along the entire axis and use easy intervals, such as 10's, 20's, 50's, and not intervals, such as 7's, 14's, etc., which make it difficult to read information off the graph.

NOTES

- Each axis must be labelled with what is shown on the axis and must include the appropriate units in brackets, for example temperature (°C), time (days), height (cm).
- Each point has an x and y co-ordinate and is plotted with a symbol which is big enough to see, for example a cross or circle.
- The points are then joined.
- With a ruler if the points lie in a straight line or you can draw a line of best fit where the number of points are distributed fairly evenly on each side of the line.
- Free-hand when the points appear to be following a curve.
- Do not start the line at the origin unless there is a data point for 0. If there is no reading for 0, then start the line at the first plotted point.
- The graph must have a clear, descriptive title which outlines the relationship between the dependent and independent variable.
- If there is more than one set of data drawn on a graph, a different symbol must be used for each set and a key or legend must define the symbols.

Graph showing change in plant height over 10 days



Bar Graphs

Bar graph is used to display the category of data and it compares the data using solid bars to represent the quantities. It is used to compare measurements between different groups. Bar graphs should be used when your data is not continuous, but rather is divided into different categories. If you counted the number of birds of different species, each species of bird would be its own category.

Bar Graphs are Used When

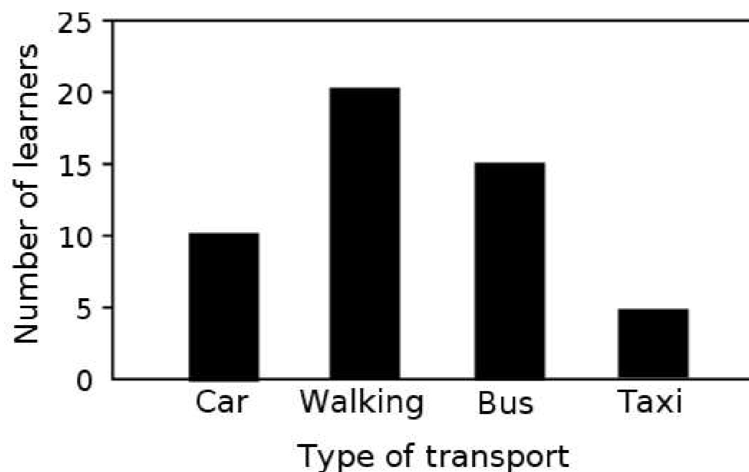
- The independent variable is discontinuous (i.e., the variables on the x-axis are each associated with something different).
- Independent variables are not numerical. For example, when examining the protein content of various food types, the order of the food types along the horizontal axis is irrelevant.

Features of Bar Graph

Bar graphs have the following features:

- The data are plotted as columns or bars that do not touch each other as each deals with a different characteristic.
- The bars must be the same width and be the same distance apart from each other.
- A bar graph can be displayed vertically or horizontally.
- A bar graph must have a clear, descriptive title, which is written beneath the graph.

  Bar graph showing how many learners use each type of transport.



Assignment

1. Plot a simple bar graph for the given data.

   Horticulture yield in various countries

Country	Yield ('000 MT/Acre)
America	1025
Sri Lanka	993
Brazil	125
France	439
India	1286

NOTES

2. Plot a multiple bar graph for the following data

   The population of the various states (X,Y, Z) of India in 2011 and 2021 are:

States	Population in 2011	Population in 2012
X	50638918	70238018
Y	69304804	81304104
Z	47817416	50019226

Histograms

The graph that uses bars to represent the frequency of numerical data that are organised into intervals. Since, all the intervals are equal and continuous, all the bars have the same width. Bar charts have their limitations; for example, they cannot be used to present continuous data. When dealing with continuous random variables a different kind of graph is required. This is called a histogram. At first sight these look similar to bar charts. There are, however, two critical differences:

- The horizontal (X-axis) is a continuous scale. As a result of this there are no gaps between the bars (unless there are no observations within a class interval);
- The height of the rectangle is only proportional to the frequency if the class intervals are all equal. With histograms it is the area of the rectangle that is proportional to their frequency.

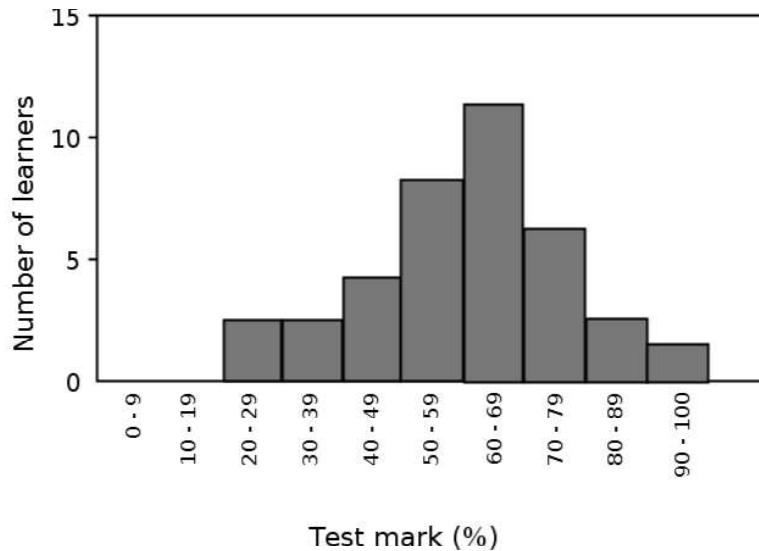
Histograms are used when the independent variable (x-axis) represents information which is continuous, such as numerical ranges, i.e., 0-9, 10-19, 20-29, etc.

Histograms have the following features:

- Unlike a bar graph, in a histogram the data are plotted as columns or bars that touch each other as they are related to each other in some way.
- The numerical categories must not overlap, for example 0-10, 10-20, 20-30, etc. The ranges must be exclusive so that there is no doubt as to where to put a reading, for example 0-9, 10-19, 20-29, etc.
- The bars can be vertically or horizontally drawn.
- A histogram must have a descriptive heading with is written below the graph.
- The axes must be labelled.

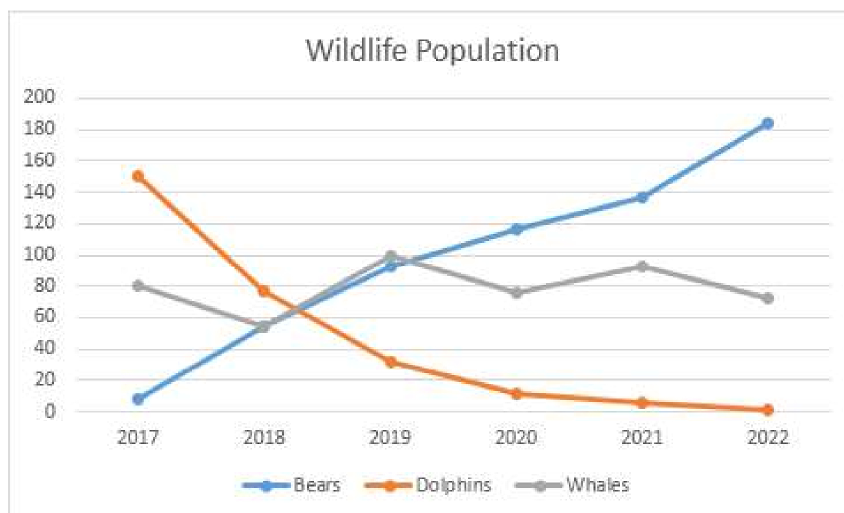
Following histogram shows the number of learners in an SS 2 Biology class with a particular percentage test score.

NOTES



Line Plot

It shows the frequency of data on a given number line. '*x*' is placed above a number line each time when that data occurs again.



Frequency Table

Frequency is a measure of the number of occurrences of a particular score in a given set of data. A frequency table is a method of organizing raw data in a compact form by displaying a series of scores in ascending or descending order, together with their frequencies—the number of times each score occurs in the respective data set. Frequency tables may be computed for both discrete and continuous variables and may take either an ungrouped or a grouped format.

A one-way frequency table shows the results of the tabulation of observations at each level of a variable. Following table illustrates the one-way tabulations of sex and race for the 40 patients. Three quarters of the patients are males, and over 87 percent of the patients are whites.

Frequency of Sex and Race of 40 Patients

Sex	Number of Patients	Percentage	Race	Number of Patients	Percentage
Male	30	75.0	White	35	87.5
Female	10	25.0	Non-white	5	12.5
Total	40	100.0	Total	40	100.0

NOTES

Two-way frequency tables, formed by the cross-tabulation of two variables, are usually more interesting than one-way tables because they show the relationship between the variables. Figure below shows the relationship between sex and body mass index where BMI has been grouped into underweight (BMI < 18.5), normal (18.5 ≤ BMI < 25), overweight (25 ≤ BMI < 30), and obese (BMI ≥ 30). The body mass index is calculated as weight in kilograms divided by height in meters squared. There are higher percentages of females in the overweight and obese categories than those found for males, but these calculations are based on very small sample sizes.

Sex			
Body Mass Index	**Male**	**Female**	**Total**
Under 18.5 (Underweight)	1 (3.3%)	0 (0.0%)	1 (2.5%)
18.5–24.9 (Normal)	10 (33.3%)	2 (20.0%)	12 (30.0%)
25.0–29.9 (Overweight)	14 (46.7%)	6 (60.0%)	20 (50.0%)
30.0 & Over (Obese)	5 (16.7%)	2 (20.0%)	7 (17.5%)
Total	30	10	40

Fig. Cross-tabulation of body mass index and sex for 40 patients in DIG40 with column percentages in parentheses

Circle Graph

Also known as the pie chart that shows the relationships of the parts of the whole. The circle is considered with 100% and the categories occupied is represented with that specific percentage like 15%, 56%, etc.

In graph theory, a circle graph is the intersection graph of a set of chords of a circle. It is an undirected graph whose vertices can be associated with chords of a circle such that two vertices are adjacent if and only if the corresponding chords cross each other. The circle graph is also called a pie graph and pie chart.

Some important formulas of circle graph are given below:

- Percentages = (Amount in the Category D Total) × 100
- Angle = (Amount in the Category D Total) × 360°

Example 1: If we ask 100 persons which TV program they like the most, we get this result.

TV Program	Persons
Grey's Anatomy (GA)	17
Family Guy (FG)	34
True Blood (TB)	15
Gossip Girl (GG)	26
America's Funniest Home Videos (AFV)	8

NOTES

We know that the total amount of persons is 100. Now we need to find the ratio for each TV program.

GA:17/100=0.17

FG:34/100=0.34

TB:15/100=0.15

GG:26/100=0.26

AFV:8100=0.08

To find the degrees of each TV program we multiply by 360.

GA: 0.17*360‘“=61.2‘“

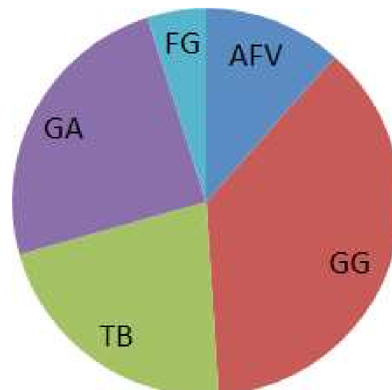
FG: 0.34*360‘“=122.4‘“

TB: 0.15*360‘“=54‘“

GG: 0.26*360‘“=93.6‘“

AFV: 0.08*360‘“=28.8‘“

And then we use a protractor to draw the chart. It will look something like this:



Example 2: The below table shows the percentage of expenses of a person in a month. Draw a circle chart for the data given.

Category	**Percentage of Expense**
Food	37%
Rent	16%
Clothing	11%
Education	20%
Medicine	4%
Other expenses	12%

Solution:

Given that;

Food = 37%

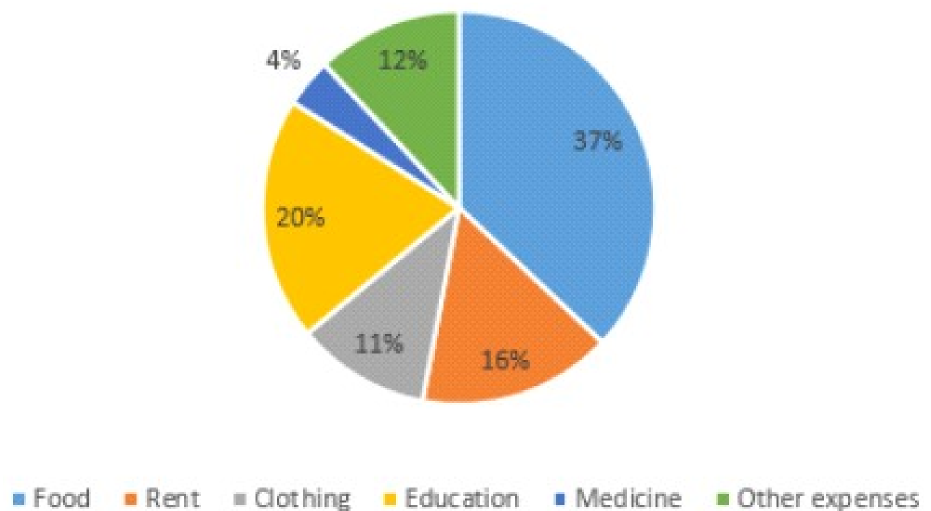
Rent = 16%

Clothing = 11%

Education = 20%

Medicine = 4%

Other expenses = 12%



General Rules for Graphical Representation of Data

There are certain rules to effectively present the information in the graphical representation. They are as follows:

- **Suitable Title:** Make sure that the appropriate title is given to the graph which indicates the subject of the presentation.
- **Measurement Unit:** Mention the measurement unit in the graph.
- **Proper Scale:** To represent the data in an accurate manner, choose a proper scale.
- **Index:** Index the appropriate colours, shades, lines and design in the graphs for better understanding.
- **Data Sources:** Include the source of information wherever it is necessary at the bottom of the graph.
- **Keep it Simple:** Construct a graph in an easy way that everyone can understand.
- **Neat:** Choose the correct size, fonts, colours, etc. in such a way that the graph should be a visual aid for the presentation of information.

EXPERIMENT II: CALCULATION OF MEAN, MEDIAN AND MODE

Aim

To calculate the mean, median and mode of the given data.

Principle

Mean, mode and median are basic statistical tools used to calculate different types of averages.

Biostatistics refers to the calculation and analysis of the data obtained in biological studies, researches or experiments. Summarizing the biological data obtained after a measurement variable requires a number for representing the 'Middle' of a set of numbers, termed as the 'Statistic of Central Tendency' or 'Statistic of Location', along with a measure of the 'Spread' of the numbers. In statistics, a central tendency or measure of central tendency is a central or typical value for a probability distribution. It is also termed as a centre or location of the distribution. Occasionally, the measures of central tendency are often termed as averages. The most common measures of central tendency are the arithmetic mean, the median and the mode. A central tendency can be calculated for either a finite set of values or for a theoretical distribution, such as the normal distribution.

Mean or Arithmetic Mean

Mean or arithmetic mean, median, and mode are the different measures of centre in a numerical data set. It summarizes a dataset with a single number to represent a 'typical/ unique' data point from the dataset.

The arithmetic mean or simply mean is the sum of the observations divided by the total number of observations. It is the most common statistic of central tendency which simply gives 'the mean' or 'the average' of the dataset. Basically, it is the 'average' number obtained by adding all data points and then dividing by the number of data points.

For example, the mean of 4, 1 and 7 is (4 + 1 + 7) / 3 = 12 / 3 = 4.

Mean is, thus, the most commonly used form of all the averages. It is the value which is obtained by dividing the aggregate of various items of the same series by the total number of observations.

Therefore, the mean or arithmetic mean is the sum of all of the data points divided by the number of data points.

Mean = Sum of Data / # of Data Points

The following is the formula:

$$\text{Mean} = \frac{\sum x_i}{n}$$

Example 1. Find the mean of the following given data: 1, 3, 4, 5, 7, 9

Solution: We first add the data as follows:

1 + 2 + 3 + 4 + 5 + 7 + 8 = 30

There are 6 data points, hence as per the formula = 30 / 6 = 5

Therefore, the mean is 5.

Calculation of Mean for Ungrouped Data

When observations are denoted by x values showing x_1, x_2, x_3… x_n, then the total number of observations is calculated by summing up the observations and dividing the sum by the total number of observations (n).

$$\bar{x} = \frac{x_1 + x_2 + x_3 + \cdots + x_n}{n}$$

Example 2: Find out the mean or average pod length of the plant from the following data.

The pod length of the ten pods of a plant are as follows:

5.2 cm 5.3 cm 5.6 cm 5.7 cm 5.4 cm

5.2 cm 5.3 cm 5.3 cm 5.4 cm 5.2 cm

Solution: The mean or average pod length of the plant is calculated as,

$$\bar{x} = \frac{\sum x}{n} = \frac{5.2+5.3+5.6+5.7+5.4+5.2+5.3+5.3+5.4+5.2}{10} cm$$

$$= \frac{53.6}{10} cm = 5.36 cm$$

Calculation of Mean for Grouped Data

When the series is discrete, each value of the variable is multiplied by their respective frequencies, and the sum of all values is divided by total number of frequencies. Variable x has the values like x_1, x_2, x_3, …, x_n and their frequencies are f_1, f_2, f_3, …, f_n, respectively.

The mean or arithmetic mean is calculated using the formula:

$$\bar{x} = \frac{f_1x_1 + f_2x_2 + f_3x_3 + \cdots + f_nx_n}{f_1 + f_2 + f_3 + \cdots + f_n} = \frac{\sum fx}{\sum f}$$

When the series is continuous, the arithmetic mean is calculated after taking the midpoint value of class intervals.

$$\bar{x} = \frac{\sum fm}{\sum f}$$

NOTES

Where,

$\bar{x}$ = Arithmetic Mean

Σfm = Sum Values of Midpoint Value Multiplied by their Frequencies

Σf = Sum of Frequencies

m = Mid Points of Various Class Intervals

Example 3: An observation on 32 Balsam plants shows the following data. Calculate the arithmetic mean.

No. of flowers/plant (x)	4	5	6	7	8	9
No. of plants (f)	3	5	6	9	5	4

Solution: We calculate the arithmetic mean as follows:

No. of flowers/plant (x)	No. of plants (f)	fx
4	3	12
5	5	25
6	6	36
7	9	63
8	5	40
9	4	6
	Σf = 32	Σfx = 212

$$\bar{x} = \frac{\sum fx}{\sum f} = \frac{212}{32} = 6.62(\text{approx})$$

The average number of flowers/plant is 6.62.

The average mean or arithmetic mean is calculated as follows:

No. of pods/plant	Mid points of class (m)	No. of plants frequency (f)	mf
15-17	16	5	80
18-20	19	6	114
21-23	22	8	176
24-26	25	12	300
27-29	28	22	616
30-32	31	18	558
33-35	34	15	510
36-38	37	9	333
39-41	40	5	200
		Σf = 100	Σmf = 2.887

$$\text{Arithmetic Mean} = \overline{x} = \frac{\sum mf}{\sum f} = \frac{2.887}{100} = 28.87.$$

NOTES

The arithmetic mean is 28.87.

Median

The median is the middle number obtained by ordering/organizing all data points and picking out the one in the middle or if there are two middle numbers, then taking the mean of those two numbers. Therefore, the median is the middle point in a dataset, i.e., half of the data points are smaller than the median and half of the data points are larger.

For example, the median of 4, 1, and 7 is 4 because when the numbers are arranged in order then we have the sequence (1, 4, 7) in which 4 is the middle number.

The median of a distribution is defined as the value of that variable which divides the total frequency into two equal parts when the series is arranged in ascending or descending order of magnitude. So in a distribution, half of the values remain below median value and half of the values remain above the median value.

Finding the Median

- Arrange the data points from smallest to largest.
- If the number of data points is odd, the median is the middle data point in the list.
- If the number of data points is even, the median is the average of the two middle data points in the list.

Example 4: Find the median of the following data:

1, 4, 2, 5, 0

Solution: First arrange the data in order form as follows:

0, 1, 2, 4, 5

Because there is an odd number of data points, hence the median is the middle data point, i.e., 2.

0, 1, 2, 4, 5

Therefore, the median is 2.

Example 5. Find the median of the following data:

10, 40, 20, 50

Solution: First arrange the data in order form as follows:

10, 20, 40, 50

NOTES

In this case, there is an even number of data points, hence the median is the average of the middle two data points, i.e., 20 and 40.

10, 20, 40, 50

Therefore,

Median = 20 + 40 / 2 = 60 / 2 = 30

The median in this case is 30.

Median Value for Ungrouped Data

Median value is the value of the n+1 / 2 item. But this formula is applicable only when item number is odd. But when the item number is even, then the median value is calculated by the mean value of n/2th and (n / 2+1)th items,

$$\therefore \text{ Median} = \frac{\frac{n}{2}\text{th value} + \left(\frac{n}{2}+1\right)\text{th value}}{2}$$

Example 6: Calculate the median number of flowers in the following observation obtained from garden plants.

Plant no.	1	2	3	4	5	6	7	8
No. of flowers	20	17	25	18	23	21	16	26

Solution: The median is calculated as follows:

Item no.	No. of flowers/plant Ascending	No. of flowers/plant Descending
1	16	26*
2	17	25
3	18	23
4	20	21
5	21	20
6	23	18
7	25	17
8	26*	16

The observations are arranged in both ascending and descending order. In case of observation of 7 plants the * marked item no. should not be considered.

If we take 7 observations, then the median value will be value of 7+1/2th, i.e., 4^{th} item, i.e., 20.

If we take 8 observations, then the median value will be the mean of 8/2th and 8/2 +1th item, i.e., 21.

Mean of 4th and 5th item, i.e., mean of 20 and 21 which is 20.5.

NOTES

Median Value for Grouped Data

For grouped data, the classes are arranged according to the ascending order and respective frequencies are written against them. The frequencies are then cumulated and position of the median is calculated by the same formula. The median value is the mid value of the class in which the median item value is placed. Consider the following table showing the class interval, mid value, frequency and cumulative frequency for number of pods.

Class interval	Mid value	Frequency	Cumulative frequency
15-17	16	5	5
18-20	19	6	11
21-23	22	8	19
24-26	25	12	31
27-29	28	22	53
30-32	31	18	71
33-35	34	15	86
36-38	37	9	95
39-41	40	5	100

Because the total number of variables is 100, hence the median value will be the value which is in between the value of 50th and 51st item value.

50^{th} and 51^{st} item value is in the class interval 27-29 (No. of Pods).

Therefore, the median value is 28 of this observation.

Mode

The mode is referred as the most frequent number occurring in the dataset, i.e., the number that occurs the highest number of times.

Therefore, the mode is the most commonly occurring data point in a dataset. The mode is useful when there are a lot of repeated values in a dataset. There can be no mode, one mode, or multiple modes in a dataset.

For example, the mode of the dataset (4, 2, 4, 3, 2, 2) is 2 because it is occurring three times, which is more than any other number.

Example 7. Find the mode of the following data:

0, 0, 1, 1, 1, 1, 1, 1, 2, 2, 2, 3, 5

Solution: The most occurring value in the dataset is,

0, 0, 1, 1, 1, 1, 1, 1, 2, 2, 2, 3, 5

Therefore, the mode is 1.

Example 8. The observation on 30 Balsam plants shows the following data. Calculate the mode from this observation.

No. of flowers/plant (x)	3	4	5	6	7	8	9	10
No. of plants (f)	1	3	2	8	5	8	2	1

Solution: Here the mode value cannot be calculated by just assessment, as the maximum frequency is observed in case of two values of variable 6 and 8. Therefore to determine the modal class, the data is grouped as follows.

If 2 values are taken together then the grouped data can be arranged in the following manner:

Class value	Mid value (m)	frequency
3-4	3.5	4
5-6	5.5	10
7-8	7.5	13
9-10	9.5	3

Here the modal class is 7-8, where mid value is 7.5, so the mode value of this distribution is 7.5. This type of distribution is called bimodal distribution.

Assignment

1. Calculate the mean, median and mode of each set of numbers given below:

(a)	4	4	9	8	5						
(b)	6	7	4	7	7	5	6	2	9		
(c)	8	4	5	3	5	7	8				
(d)	1	6	7	7	4	9	1	7	10	1	

2. The table below gives the number of accidents each year (in the month of January) on Yamuna Expressway, India:

2010	2011	2012	2013	2014	2015	2016	2017
15	20	18	22	33	18	14	5

   (a) Calculate the mean, median and mode for the values above.

   (b) NHAI plans to make this expressway safer. Which measure will they use for this purpose?

EXPERIMENT III: CALCULATION OF STANDARD DEVIATION, STANDARD ERROR AND ANOVA (ONE WAY AND TWO WAY)

Aim

To calculate the standard deviation and standard error of the given data.

The Standard Deviation (SD) is a statistic that measures the dispersion of a dataset relative to its mean and is calculated as the square root of the variance. Basically, it is calculated as the square root of variance by determining the variation between each data point relative to the mean. If the data points are further from the mean, there is a higher deviation within the dataset; thus, the more spread out the data, the higher the standard deviation.

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Standard deviation is the most commonly used measure of dispersion of data around a mean - described more frequently than the variance. Arithmetically, standard deviation is defined as the square root of the variance.

The Standard Deviation (SD) is a measure of how spread out numbers are.

The symbol for Standard Deviation is σ (the Greek letter sigma) and the formula for

Standard Deviation is:

$$\sigma = \sqrt{\frac{1}{N}\sum_{i=1}^{N}(x_i - \mu)^2}$$

Say we have a bunch of flowers represented using numbers as 9, 2, 5, 4, 12, 7, 8, 11.

To calculate the Standard Deviation of these numbers:

- Calculate the Mean (the simple average of the numbers).
- Then for each number, subtract the mean and square the result.
- Then calculate the Mean of those squared differences.
- Take the square root to obtain the result.

Example 1: Assume that there are 20 rose bushes and the number of flowers on each bush is, 9, 2, 5, 4, 12, 7, 8, 11, 9, 3, 7, 4, 12, 5, 4, 10, 9, 6, 9, 4.

Calculate the Standard Deviation.

Solution: The Standard Deviation is calculated as follows:

Step 1. Calculate the mean.

In the formula above μ is the mean of all the values.

For example, we have the dataset 9, 2, 5, 4, 12, 7, 8, 11, 9, 3, 7, 4, 12, 5, 4, 10, 9, 6, 9, 4.

The mean for this is:

9 + 2 + 5 + 4 + 12 + 7 + 8 + 11 + 9 + 3 + 7 + 4 + 12 + 5 + 4 + 10 + 9 + 6 + 9 + 4 / 20 = 140 / 20 = 7

Therefore, μ = 7

Step 2. Then for each number: Subtract the Mean and Square the result.

As per the formula,

$(x^i - \mu)^2$

Here, x_i refers to the individual values of x which are 9, 2, 5, 4, 12, 7, etc.

In other words, $x_1 = 9$, $x_2 = 2$, $x_3 = 5$, and so on.

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For each value, subtract the mean and square the result as follows:

$(9 - 7)^2 = (2)^2 = 4$

$(2 - 7)^2 = (-5)^2 = 25$

$(5 - 7)^2 = (-2)^2 = 4$

$(4 - 7)^2 = (-3)^2 = 9$

$(12 - 7)^2 = (5)^2 = 25$

$(7 - 7)^2 = (0)^2 = 0$

$(8 - 7)^2 = (1)^2 = 1$

And so on.

The final result is:

4, 25, 4, 9, 25, 0, 1, 16, 4, 16, 0, 9, 25, 4, 9, 9, 4, 1, 4, 9

Step 3. Then calculate the mean of those squared differences.

To calculate the mean, add up all the values then divide by how many.

First add up all the values from the previous step using 'Sigma' - ".

To add up all the values from 1 to N, where N = 20 in this case because there are 20 values:

$$\sum_{i=1}^{N}(x_i - \mu)^2$$

Which means that sum all values from $(x_1-7)^2$ to $(x_N - 7)^2$

We have already calculated $(x_1-7)^2 = 4$ in the previous step. Therefore, to sum them up:

= 4 + 25 + 4 + 9 + 25 + 0 + 1 + 16 + 4 + 16 + 0 + 9 + 25 + 4 + 9 + 9 + 4 + 1 + 4 + 9

= 178

But this is not yet the mean. To obtain the mean, we have multiply by 1/N (the same as dividing by N):

$$\frac{1}{N}\sum_{i=1}^{N}(x_i - \mu)^2$$

Mean of Squared Differences = (1/20) × 178 = 8.9

This value is called the 'Variance'.

Step 4. Taking the square root.

Therefore the Standard Deviation 'σ' = " (8.9) = 2.983.

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Standard Error of the Mean (SEM)

The Standard Deviation (SD) measures the amount of variability, or dispersion, for a subject set of data from the mean, while the Standard Error of the Mean (SEM) measures how far the sample mean of the data is likely to be from the true mean. The SEM is always smaller than the SD. Both the 'Standard Deviation' and 'Standard Error (SE)' are often used in experimental studies. In these studies, the Standard Deviation (SD) and the estimated Standard Error of the Mean (SEM) are used to present the characteristics of sample data and to explain statistical analysis results. Alternatively, SD indicates how accurately the mean represents sample data. However, the meaning of SEM includes statistical inference based on the sampling distribution. SEM is the SD of the theoretical distribution of the sample means (the sampling distribution).

Calculating Standard Error of the Mean

Standard Deviation $\sigma = \sqrt{\frac{\sum_{i=1}^{n}(x_i - \bar{x})^2}{n-1}}$

Variance = σ^2

Standard Error

$$(\sigma_{\bar{x}}) = \frac{\sigma}{\sqrt{n}}$$

Where,

x = Sample Mean

n = Sample Size

Therefore, the Standard Error of the Mean (SEM) is calculated by taking the standard deviation and dividing it by the square root of the sample size.

The formula for the SD includes the following steps:

Step 1: Take the square of the difference between each data point and the sample mean, finding the sum of those values.

Step 2: Divide that sum by the sample size minus one, which is the variance.

Step 3: Take the square root of the variance to obtain the SD.

Standard error validates the accuracy of a single sample or of the multiple samples by analysing deviation within the means. The SEM defines how precise the mean of the sample is vs. the true mean of the dataset. As the size of the sample data grows larger, the SEM decreases vs. the SD.

The standard error is defined as the measure of descriptive statistics. It represents the standard deviation of the mean within a dataset. Thus, it functions

as a measure of variation for random variables, providing a measurement for the spread. The smaller the spread, the more accurate the dataset.

Assignment

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1. The number of pots made by a potter over the week are as follows: calculate the mean and standard deviation and standard error.

Monday	
Tuesday	
Wednesday	
Thursday	
Friday	
Saturday	
Sunday	

Observation	Value (x)	Deviation ($x - \bar{x}$)	Standard Deviation $(x - \bar{x})^2$
1			
2			
3			
4			
5			
6			
7			
Total		0	

2. The average daily earnings of candyfloss seller (who sells at variable rates) are Rs 12 with a population standard deviation of Rs. 3. Given the total number of candyfloss sold (sample size) as 30, estimate and interpret the standard error of the sample mean.

   (Ans Rs. 0.55)

3. The sample of 30 latest returns on India bulls stock shows a mean return of Rs 288 with a sample standard deviation of Rs 9.36. Estimate the standard error of the sample mean.

   (Ans Rs 1.44)

4. The average daily temperature (for 6 days of the week) of Delhi in the month of March has been recorded as follows:

Monday	19
Tuesday	21
Wednesday	25
Thursday	28
Friday	27
Saturday	26

Calculate the mean and standard deviation of the given data.

ANOVA (One Way and Two Way)

Aim

To calculate ANOVA (One Way and Two Way) of the given data.

ANalysis Of VAriance (ANOVA) is a collection of statistical models and their associated estimation procedures, such as the 'variation' among and between groups, used to analyse the differences among group means in a sample. ANOVA was developed by statistician and evolutionary biologist Ronald Fisher. The ANOVA is based on the law of total variance, where the observed variance in a particular variable is partitioned into components attributable to different sources of variation. In its simplest form, ANOVA provides a statistical test of whether the population means of several groups are equal, and therefore generalizes the t-test to more than two groups. ANOVA is useful for comparing/testing three or more group means for statistical significance.

The ANOVA is a parametric statistical technique used to compare datasets. This technique was invented by R. A. Fisher, and is thus often also referred to as Fisher's ANOVA. It is similar in application to techniques, such as t-test and z-test, in that it is used to compare means and the relative variance between them. However, ANOVA is best applied where more than 2 populations or samples are meant to be compared.

The use of the ANOVA parametric statistical technique involves certain key assumptions, including the following:

- **Independence of Case:** Independence of case assumption means that the case of the dependent variable should be independent or the sample should be selected randomly. There should not be any pattern in the selection of the sample.
- **Normality:** Distribution of each group should be normal. The Kolmogorov- Smirnov or the Shapiro-Wilk test may be used to confirm normality of the group.
- **Homogeneity:** Homogeneity means variance between the groups should be the same. Levene's test is used to test the homogeneity between groups.

  If particular data follows the above assumptions, then the ANOVA is the best technique to compare the means of two, or more, populations.

The analysis of ANOVA has following three types:

- **One Way Analysis:** When we are comparing more than three groups based on one factor variable, then it said to be One Way ANOVA. For

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example, if we want to compare whether or not the mean output of three workers is the same based on the working hours of the three workers.

- **Two Way Analysis:** When factor variables are more than two, then it is said to be Two Way ANOVA. For example, based on working condition and working hours, we can compare whether or not the mean output of three workers is the same.
- **K-Way Analysis:** When factor variables are K, then it is said to be the K-Way ANOVA.

The ANOVA is computed using the following key concepts:

- **Sum of Square between Groups:** For the sum of the square between groups, we calculate the individual means of the group, then we take the deviation from the individual mean for each group. And finally, we will take the sum of all groups after the square of the individual group.
- **Sum of Squares within Group:** In order to get the sum of squares within a group, we calculate the grand mean for all groups and then take the deviation from the individual group. The sum of all groups will be done after the square of the deviation.
- **F –Ratio:** To calculate the F-ratio, the sum of the squares between groups will be divided by the sum of the square within a group.
- **Degree of Freedom:** To calculate the degree of freedom between the sums of the squares group, we will subtract one from the number of groups. The sum of the square within the group's degree of freedom will be calculated by subtracting the number of groups from the total observation.
- **BSS df = (g-1)** for BSS is Between the Sum of Squares, where g is the group, and df is the degree of freedom.
- **WSS df = (N-g)** for WSS is Within the Sum of Squares, where N is the total sample size, and df is the degree of freedom.
- **Significance:** At a predetermine level of significance (usually at 5%), we will compare and calculate the value with the critical table value. Today, however, computers can automatically calculate the probability value for F-ratio suing the statistical software.

Assignment

1. A pilot study was conducted to examine the impact of vitamin supplements in meals on IQ test of the teenagers (both boys and girls). 50% of the

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teenagers were provided with the vitamin supplements and remaining without supplements. The results of IQ test (10 point scale) are given below:

Group	With Vitamin Supplement	Without Vitamin Supplement
Boys	10	5
	7	4
	9	7
	6	4
	8	5
Girls	5	3
	4	4
	6	5
	3	1
	2	2

Complete the following ANOVA table and interpret your answer (you can use MS-Excel, SPSS or Minitab or any other software).

Source	SS	df	MS	F
Vitamin supplement	20	1		
Gender	45	1		
Vitamin supplement x Gender	5	1		
Within	36	16		
Total				

Answer:

Source	SS	df	MS	F
Vitamin supplement	20	1	20.00	8.89
Gender	45	1	45.00	20.00
Vitamin supplement x Gender	5	1	5.00	2.22
Within	36	16	2.25	
Total	106	19		

2. During COVID-19 pandemic, a study examining differences in stress management in between teenagers, adults and elderly (males and females)

was conducted in Mumbai. Each participant answered to the questionnaire provided to them. A high score (10-point scale) indicates better stress management, as shown in the given table.

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Group	Teenagers	Adults	Elderly
Male	4 2 3 4 2	7 5 7 5 6	10 7 9 8 11
Female	7 4 3 6 5	8 10 7 7 8	10 9 12 11 13

Complete the following ANOVA table and interpret your answer.

Source	SS	df	MS	F
Age group	180	2		
Gender	30	1		
Age group x Gender	0	2		
Within	44	24		
Total				

Answer:

Source	SS	df	MS	F
Age group	180	2	90.00	49.09
Gender	30	1	30.00	16.36
Age group x Gender	0	2	0.00	0.00
Within	44	24	1.83	
Total	254	29		

3. A clinical trial was conducted to observe the effect of oral drug for covid-19 pandemic. The subjects were randomly allocated to anyone group viz.: a placebo group (no drug administered, only vitamin supplements), a group

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that received a low dose of the oral drug, and a group that received a moderate dose of the oral drug. The presence of antibodies against the virus were detected as per the data given the table below:

Placebo	Low Dose	Moderate Dose
38	22	14
47	19	26
39	8	11
25	23	18
42	31	5

Calculate ANOVA and interpret your answer.

Answer:

Source	SS	df	MS	F
Between	1484.9333333	2	742.4666666	11.26
Within	790.8	12	65.9	
Total	2275.733333	14		

EXPERIMENT IV: CHI-SQUARE TEST, T-TEST, REGRESSION AND CORRELATION

Aim

To calculate and interpret the given problems using Chi square test.

A Chi-Squared Test, also written as χ^2 test, is any statistical hypothesis test where the sampling distribution of the test statistic is a chi-squared distribution when the null hypothesis is true. Without other prerequisite, the 'chi-squared test' often is used as short for Pearson's chi-squared test. The chi-squared test is used to determine whether there is a significant difference between the expected frequencies and the observed frequencies in one or more groups/categories.

In the standard applications of this test, the observations are classified into mutually exclusive classes, and there is some theory, or say null hypothesis, which gives the probability that any observation falls into the corresponding class. The purpose of the test is to evaluate how likely the observations that are made would be, assuming the null hypothesis is true.

Chi-squared tests are often constructed from a sum of squared errors, or through the sample variance. Test statistics that follow a chi-squared distribution

arise from an assumption of independent normally distributed data, which is valid in many cases due to the central limit theorem. A chi-squared test can be used to attempt rejection of the null hypothesis that the data are independent.

Also considered a chi-squared test is a test in which this is asymptotically true, meaning that the sampling distribution (if the null hypothesis is true) can be made to approximate a chi-squared distribution as closely as desired by making the sample size large enough. Figure below illustrates the Chi-squared distribution, showing χ^2 on the x-axis and p-value on the y-axis.

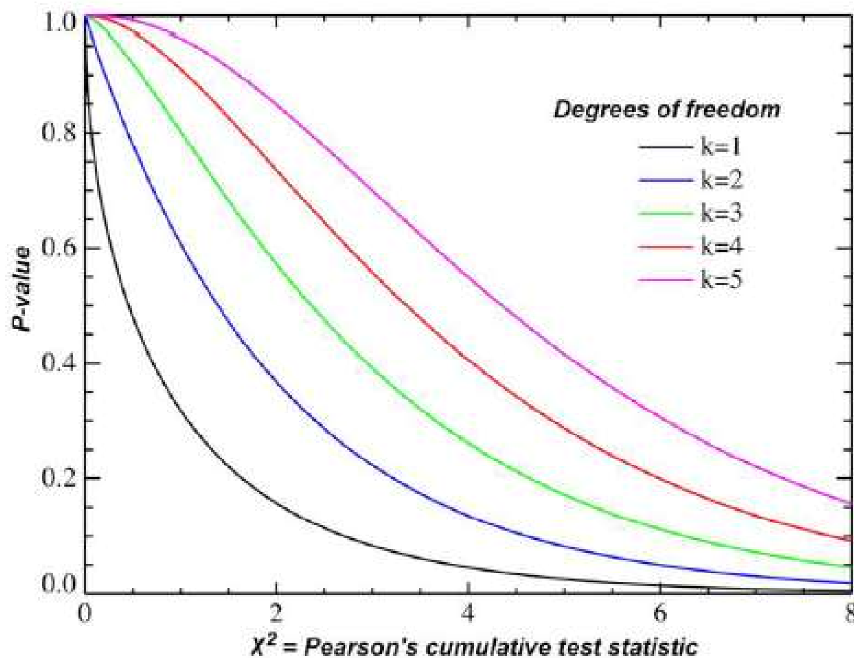


Fig. 1 Chi-Squared Distribution

In the 19th century, statistical analytical methods were mainly applied in biological data analysis and it was customary for researchers to assume that observations followed a normal distribution. Until the end of 19th century, Pearson noticed the existence of significant skewness within some biological observations. In order to model the observations regardless of being normal or skewed, Pearson formulated the Pearson distribution, a family of continuous probability distributions, which includes the normal distribution and many skewed distributions, and proposed a method of statistical analysis consisting of using the Pearson distribution to model the observation and performing the test of goodness of fit to determine how well the model and the observation really fit.

The chi-squared distribution is continuous probability distribution whose shape is defined by the number of degrees of freedom. It is a right-skew distribution, but as the number of degrees of freedom increases it approximates the Normal distribution, as shown in Figure below. The chi-squared distribution is important for its use in chi-squared tests. These are often used to test deviations between

observed and expected frequencies, or to determine the independence between categorical variables. When conducting a chi-squared test, the probability values derived from chi-squared distributions can be looked up in a statistical table. Figure below illustrates the chi-squared distribution for various degrees of freedom (df). The distribution becomes less right-skew as the number of degrees of freedom increases.

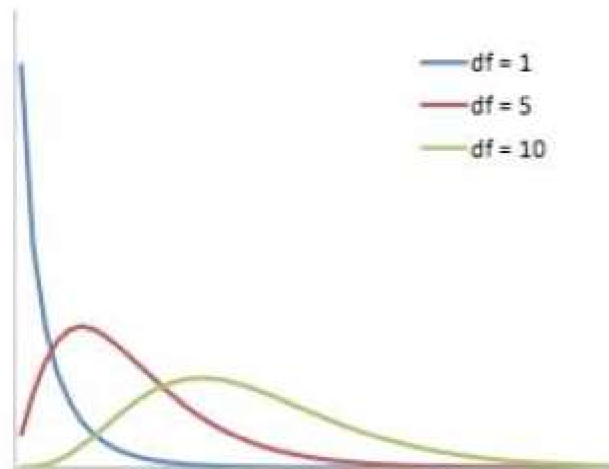


Fig. 2 *Chi-Squared Distribution for Various Degrees of Freedom*

There are two types of chi-square tests. Both use the chi-square statistic and distribution for different purposes:

- A chi-square goodness of fit test determines if a sample data matches a population.
- A chi-square test for independence compares two variables in a contingency table to see if they are related. In a more general sense, it tests to see whether distributions of categorical variables differ from each another.
  - A very small chi square test statistic means that your observed data fits your expected data extremely well. In other words, there is a relationship.
  - A very large chi square test statistic means that the data does not fit very well. In other words, there is not a relationship.

A chi-square test will give a p-value. The p-value defines that the test results are significant or not. In order to perform a chi-square test and get the p-value, the following information is required:

- Degrees of freedom. That is just the number of categories minus 1.
- The alpha level (α). This is chosen by the experimenter/researcher. The usual alpha level is 0.05 (5%), but you could also have other levels like 0.01 or 0.10.

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Assignment

1. In a lotto, the following cards (drawn at random from an infinite deck), were counted:

Spades	404
Hearts	420
Diamonds	400
Clubs	376
Total	1600

   Could it be that the suits are equally likely? Or are these inconsistencies too much to be random?

   (Ans: chi-square $\rightarrow$ 2.480; critical value $\rightarrow$ 7.815)

2. In a lotto, the following cards (drawn at random from an infinite deck), were counted:

Spades	404
Hearts	420
Diamonds	400
Jokers	82
Clubs	356
Total	1662

   a. Could it be that the suits are equally likely? Or are these inconsistencies too much to be random?

   b. How many jokers would you expect out of 1662 random cards? How many of each suit?

   c. Is it possible that the cards are really random? Or are the discrepancies too large?

   (Ans: chi-square $\rightarrow$ 12.680, critical value $\rightarrow$ 9.488)

3. In a genetic engineering experiment, a scientist was attempting to cross orange marigold flowers and a yellow marigold flowers. She predicted a phenotypic outcome of the traits in terms of the following ratios:

   Orange marigold flowers only

   Yellow marigold flowers only

   Both orange and yellow marigold flowers

The following were the results after the experiment:

   Orange marigold flowers only: 50 flowers

   Yellow marigold flowers only: 41 flowers

   Both combined orange and yellow marigold flowers: 85

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According to the Chi-square test, did she get the predicted outcome?

(Hint: Since $X_c^2 = \sum \frac{(O_i - E_i)^2}{E_i} = 4.74 < 5.991$, null hypothesis accepted).

Student *t*-Test

Aim

To perform *t*-test.

Principle: The *t*-test is any statistical hypothesis test in which the test statistic follows a Student's *t* distribution under the null hypothesis. The *t*-statistic was introduced in 1908 by William Sealy Gosset, a chemist working for the Guinness brewery in Dublin, Ireland. 'Student' was his pen name, so this test is also termed as 'Student *t*-Test'.

A *t*-test is most commonly applied when the test statistic would follow a normal distribution if the value of a scaling term in the test statistic were known. When the scaling term is unknown and is replaced by an estimate based on the data, the test statistics (under certain conditions) follow a Student's *t* distribution. The *t*-test can be used, for example, to determine if the means of two sets of data are significantly different from each other.

Among the most frequently used *t*-tests are:

- A one-sample location test of whether the mean of a population has a value specified in a null hypothesis.
- A two-sample location test of the null hypothesis such that the means of two populations are equal. All such tests are usually called Student's *t*-tests, though strictly speaking that name should only be used if the variances of the two populations are also assumed to be equal, the form of the test used when this assumption is dropped is sometimes called Welch's *t*-test. These tests are often referred to as 'unpaired' or 'independent samples' *t*-tests, as they are typically applied when the statistical units underlying the two samples being compared are non-overlapping.

The test statistics have the form $t = Z/s$, where Z and s are functions of the data.

Z may be sensitive to the alternative hypothesis, i.e., its magnitude tends to be larger when the alternative hypothesis is true, whereas s is a scaling parameter that allows the distribution of *t* to be determined.

As an example, in the one-sample *t*-test,

$$t = \frac{Z}{s} = \frac{\overline{X} - \mu}{\hat{\sigma}/\sqrt{n}}$$

Where,

- X is the sample mean from a sample $X_1, X_2, \ldots, X_n$, of size n.
- s is the standard error of the mean.
- $\hat{\sigma}$ is the estimate of the standard deviation of the population.
- μ is the population mean.

The assumptions underlying a *t*-test in its simplest form are that:

- X follows a normal distribution with mean μ and variance σ^2/n.
- s^2 follows a χ^2 distribution with $n - 1$ degrees of freedom.
- Z and s are independent.

t-Distribution

Student's *t*-distribution is a continuous probability distribution with a similar shape to the normal distribution but with wider tails. The *t*-distributions are used to describe samples which have been drawn from a population, and the exact shape of the distribution varies with the sample size. The smaller the sample size, the more spread out the tails, and the larger the sample size, the closer the *t*-distribution is to the Normal distribution, as shown in Figure below. Whilst in general the Normal distribution is used as an approximation when estimating means of samples from a normally distribution population, when the same size is small (say $n < 30$), then the *t*-distribution should be used in preference. Figure 3 illustrates the *t*-distribution for various sample sizes. As the sample size increases, then the *t*-distribution more closely approximates the normal.

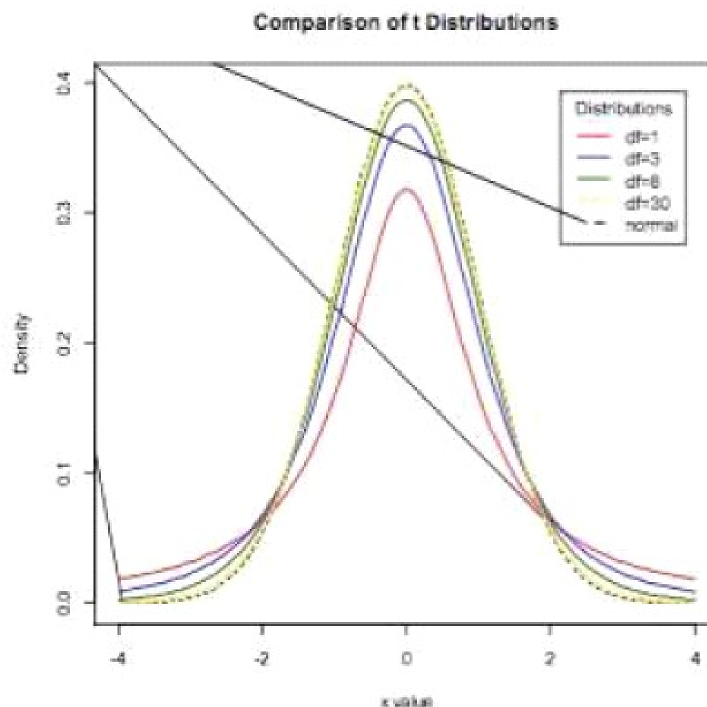


Fig. 3 *Comparison of the* t*-Distribution for Various Sample Sizes*

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Assignment

1. In a cross sectional study to understand the differences between Male and Female on the knowledge about consumer awareness. Ten males and ten females were given a consumer awareness questionnaire. Scores on the measure range from 0 to 60 with high scores indicative of high consumer awareness; low scores indicative of low consumer awareness; as shown in the data given below: Compute the appropriate *t*-test.

Male	Female
45	34
38	22
52	15
48	27
25	37
39	41
51	24
46	19
55	26
46	36
Mean =	Mean =
S =	S =
S^2 =	S^2 =

(i) What is your computed answer?

(ii) What would be the null hypothesis in this study?

(iii) What would be the alternate hypothesis?

(iv) What probability level did you choose and why?

(v) What is your t_{crit}?

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(vi) Is there a significant difference between the two groups?

(vii) Interpret your answer.

(viii) If you have made an error, would it be a Type I or a Type II error? Explain your answer.

Answer:

Male	Female
45	34
38	22
52	15
48	27
25	37
39	41
51	24
46	19
55	26
46	36
Mean = 44.5	Mean = 28.1
S = 8.682677518	S = 8.543353492
S^2 = 75.388888888	S^2 = 72.988888888

1. The value of $t_{obs} = 4.257$
2. Null hypothesis: There are no significant differences between male and female on consumer awareness

   Alternate hypothesis: Consumer awareness scores of males and females are different.

   At Probability level 0.05; $t_{crit} = 2.101$
3. The t_{obs} is in the tail, therefore there is a significant difference between the two groups. The males have significantly greater knowledge on consumer awareness than females ($t = 4.257$, $p < .001$).

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Linear Regression and Correlation

Aim

To understand linear regression and correlation plot graph for the given problems.

Linear Regression

Regression analysis is a set of statistical processes for estimating the relationships among variables. It includes many techniques for modelling and analysing several variables, when the focus is on the relationship between a dependent variable and one or more independent variables. More specifically, regression analysis helps one understand how the typical value of the dependent variable changes when any one of the independent variables is varied, while the other independent variables are held fixed.

Definition: Regression is a statistical measurement used in finance, investing and other disciplines that attempts to determine the strength of the relationship between one dependent variable (usually denoted by Y) and a series of other changing variables (known as independent variables).

Regression analysis is a set of statistical methods used for the estimation of relationships between a dependent variable and one or more independent variables. It can be utilized to assess the strength of the relationship between variables and for modelling the future relationship between them. The regression analysis includes several variations, such as linear, multiple linear, and nonlinear. The most common models are simple linear and multiple linear.

Non-linear regression analysis is commonly used for more complicated data sets in which the dependent and independent variables show a non-linear relationship.

Linear Model Assumptions for Regression Analysis

Linear regression analysis is based on following six fundamental assumptions:

- The dependent and independent variables show a linear relationship between the slope and the intercept.
- The independent variable is not random.
- The value of the residual (error) is zero.
- The value of the residual (error) is constant across all observations.
- The value of the residual (error) is not correlated across all observations.
- The residual (error) values follow the normal distribution.

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Simple Linear Regression

Simple linear regression is a model that assesses the relationship between a dependent variable and one independent variable. The simple linear model is expressed using the following equation:

Y = a + bX + €

Where:

Y = Dependent Variable

X = Independent (Explanatory) Variable

a = Intercept

b = Slope

€ = Residual (Error)

Multiple Linear Regression

Multiple linear regression analysis is essentially similar to the simple linear model, with the exception that multiple independent variables are used in the model. The mathematical representation of multiple linear regression is:

$Y = a + bX_1 + cX_2 + dX_3 + €$

Where:

Y = Dependent Variable

X_1, X_2, X_3 = Independent (Explanatory) Variables

a = Intercept

b, c, d = Slopes

€ = Residual (Error)

Multiple linear regression follows the same conditions as the simple linear model. However, since there are several independent variables in multiple linear analysis, there is another mandatory condition for the model named as Non-Collinearity.

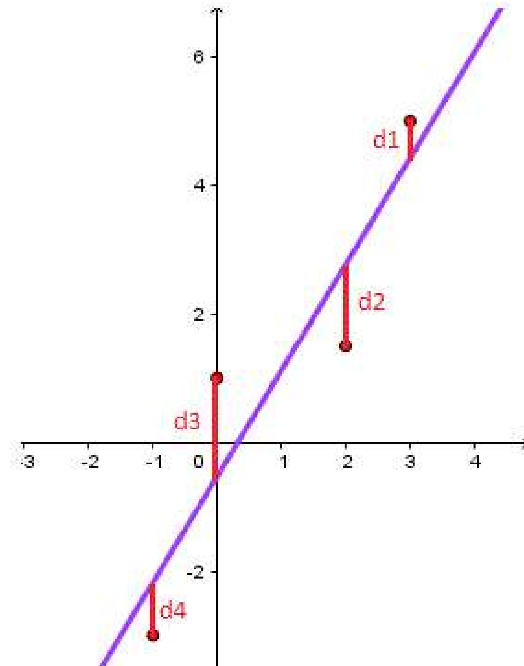
Non-Collinearity: Independent variables should show a minimum of correlation with each other. If the independent variables are highly correlated with each other, it will be difficult to assess the true relationships between the dependent and independent variables.

Regression takes a group of random variables, thought to be predicting Y, and tries to find a mathematical relationship between them. This relationship is typically in the form of a straight line (linear regression) that best approximates all the individual data points. In multiple regression, the separate variables are differentiated by using numbers with subscripts.

NOTES

If the plot of n pairs of data (x , y) for an experiment appear to indicate a 'linear relationship' between y and x, then the method of least squares may be used to write a linear relationship between x and y.

The least squares regression line is the line that minimizes the sum of the squares (d1 + d2 + d3 + d4) of the vertical deviation from each data point to the line.



The least square regression line for the set of *n* data points is given by the equation of line in slope intercept form:

Y=a x + b

Where, a and b are given by:

$$a = \frac{n\sum_{i=1}^{n} x_i y_i - \sum_{i=1}^{n} x_i \sum_{i=1}^{n} y_i}{n\sum_{i=1}^{n} x_i^2 - (\sum_{i=1}^{n} x_i)^2}$$

$$b = \frac{1}{n}(\sum_{i=1}^{n} y_i - a\sum_{i=1}^{n} x_i)$$

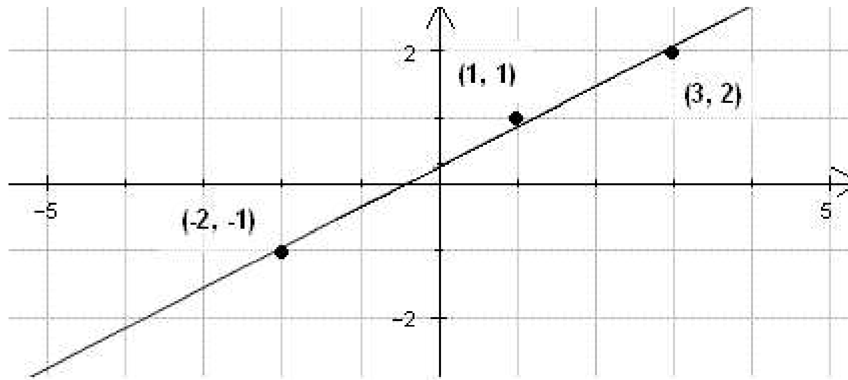
NOTES

Assignment

1. Consider the following set of points: {(-2, -1) , (1 , 1) , (3 , 2)}

   Find the least square regression line for the given data points and plot them and the regression line in the same rectangular system of axes.

   Hint: a=23/38, b=5/19



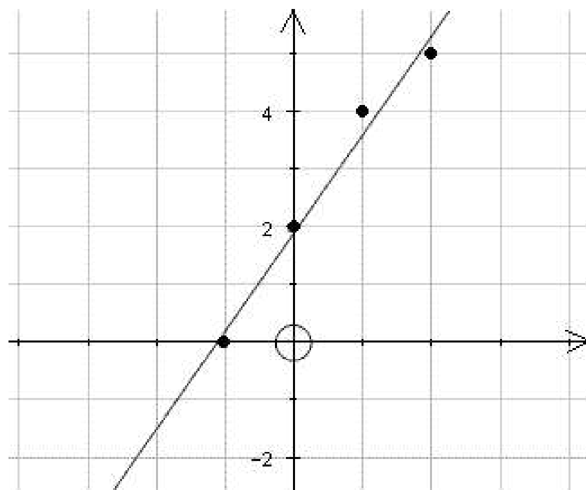
2. Find the least square regression line for the following set of data

   {(-1 , 0),(0 , 2),(1 , 4),(2 , 5)}

   Plot the given points and the regression line in the same rectangular system of axes.

   Hint:

   (a = 1.7, b = 1.9)



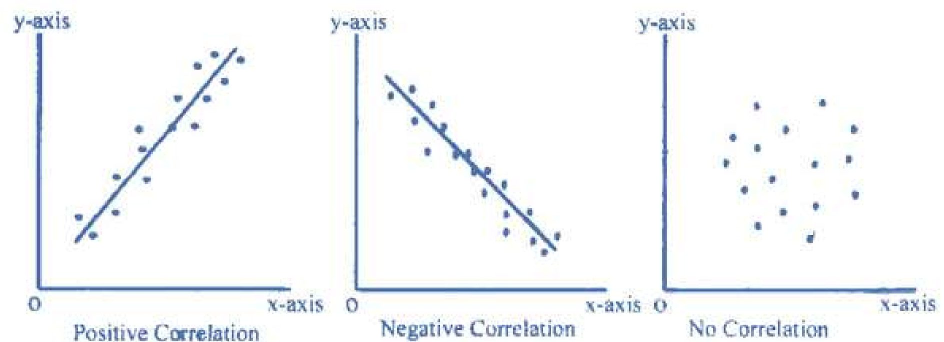
NOTES

Correlation

Methods of correlation summarize the relationship between two variables in a single number called the correlation coefficient. The correlation coefficient is usually represented using the symbol r, and it ranges from -1 to $+1$.

A correlation coefficient quite close to 0, but either positive or negative, implies little or no relationship between the two variables. A correlation coefficient close to plus 1 means a positive relationship between the two variables, with increases in one of the variables being associated with increases in the other variable.

A correlation coefficient close to -1 indicates a negative relationship between two variables, with an increase in one of the variables being associated with a decrease in the other variable. A correlation coefficient can be produced for ordinal, interval or ratio level variables, but has little meaning for variables which are measured on a scale which is no more than nominal.



The formula for Correlation is,

$$r = \frac{\sum (X - \bar{X})(Y - \bar{Y})}{\sqrt{\sum (X - \bar{X})^2} \sqrt{(Y - \bar{Y})^2}}$$

Where,

r = Correlation Coefficient

X = Average of Observations of Variable X

3 = Average of Observations of Variable Y

A correlation between variables indicates that as one variable changes in value, the other variable tends to change in a specific direction. The value of one variable can be used to predict the value of the other variable. For example, height and weight are correlated, hence as height increases the weight also tends to increase.

Scatterplot diagram helps to check for relationships between pairs of continuous data. The scatterplot shown in Figure 4 displays the height and weight of teenage girls. Each dot on the graph represents an individual girl and her height and weight on the representative axis.

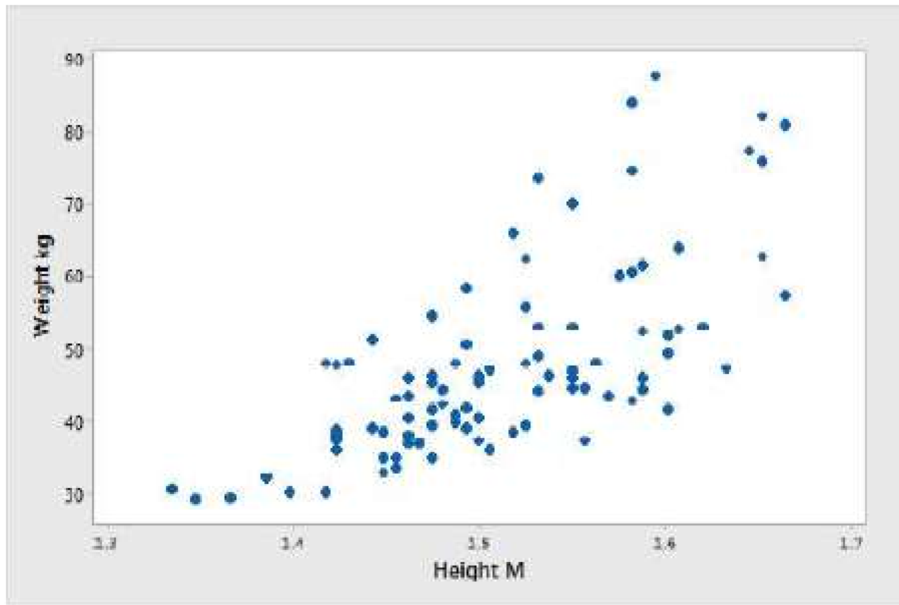


Fig. 4 *Scatterplot of the Height and Weight of Teenage Girls*

Figure 4 shows that there is a relationship between height and weight. As height increases, the weight may also increase. However, this is not a perfect relationship, for example when a specific height, say 1.5 meters, is considered then there is a range of weights associated with this specific height. However, the common tendency that height and weight increase together is unquestionably observed. Pearson's correlation takes all of the data points on this graph and represents them as a single number.

Assignment

Scientists are determining if there is a relationship between psychological positivity and immunity in the following study. Participants of the study completed a psychological positivity scale (High Score = High Psychological Positivity) and also responded to questions for measuring degree of immunity (High Score = Greater Immunity); the following data was obtained.'

NOTES

Psychological positivity	Immunity
38	4
42	3
29	11
31	5
28	9
15	6
24	14
17	9
19	10
11	15
8	19
19	17
3	10
14	14
6	18

Hint: $r^2 = 0.48$

Compute and conclude is there any relationship between psychological positivity and immunity?